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(21) International Application Number: PCT/US96/19900 (22) International Filing Date: 12 December 1996 (12.12.96) (30) Priority Data: 08/570,761 12 December 1995 (12.12.95) US (71) Applicant: CALIFORNIA INSTITUTE OF TECHNOLOGY [US/US]; 1201 East California Boulevard, Pasadena, CA 91125 (US). (72) Inventors: MEADE, Thomas, J.; 1656 New York Drive, Altadena, CA 91001 (US). TAKEUCHI, Toshihiko; 1279 Page Street, San Francisco, CA 94111 (US). GRAY, Harry, B.; 1415 E. California Boulevard, Pasadena, CA 91106 (US). SIMON, Melvin; 1075 Old Mill Road, San Marino, CA 91108 (US). LOUIE, Angelique, Y.; 808 Magnolia #6, Pasadena, CA 91106 (US). (74) Agents: GOLDBERG, Jules, E. et al.; McAulay Fisher Nissen Goldberg & Kiel, L.L.P., 261 Madison Avenue, New York, NY 10016-2391 (US).		(81) Designated States: AU, CA, IL, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: COBALT SCHIFF BASE COMPOUNDS (57) Abstract <p>The invention relates to cobalt compounds in which divalent or trivalent cobalt is complexed with water soluble tetradentate Schiff's bases. The tetradentate Schiff's bases preferably contain two nitrogen atoms and two oxygen atoms as coordinating atoms. The compounds can contain polypeptide or nucleic acid targeting moieties and can be used to inhibit enzymes such as thrombin and to inhibit zinc finger proteins.</p> <div data-bbox="730 1155 1006 1428" style="text-align: center;"></div>		

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COBALT SCHIFF BASE COMPOUNDS

FIELD OF THE INVENTION

The invention relates to cobalt compounds, and methods of using such compounds to reduce the biological activity of proteins.

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BACKGROUND OF THE INVENTION

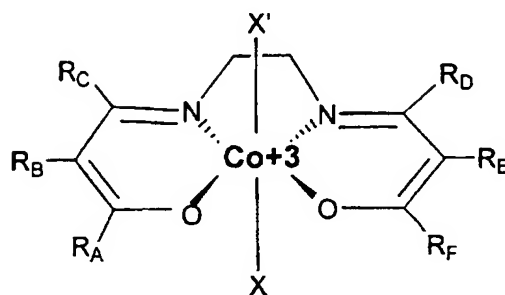
The use of metals in medicine has grown impressively in recent years as the result of a greatly advancing understanding of the structures of biologically active metal complexes and metal-containing proteins.

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Currently, a class of cobalt-containing complexes, where the cobalt is Co(III), has been shown to have antiviral, antitumor and antimicrobial activities, as well as showing use in the treatment of inflammation and burns (see U.S. Patent Nos. 4,866,054, 4,866,053, 5,049,557, 5,106,841, 5,142,076, and 5,210,096, and Wooley et al., Agents and Actions 35:274 (1992)).

-2-

These complexes have a basic core structure shown below:



These complexes are hypothesized to be active-oxygen or superoxide antagonists, thus suppressing medical conditions associated with free radicals such as inflammation.

- 5 Additionally, a Co(II) complex of isopropyl salicylic acid has been made and reported to be cytotoxic. (Ranford et al., J. Chem. Soc. Dalton Trans. (1993) 3393).

- 10 Finally, the oxidation of certain Co(III) complexes containing coordinated nitrogen mustards causes the release of activated aliphatic mustards which can act as diffusible cytotoxins. (Ware et al., J. Med. Chem. 36:1839 (1993)).

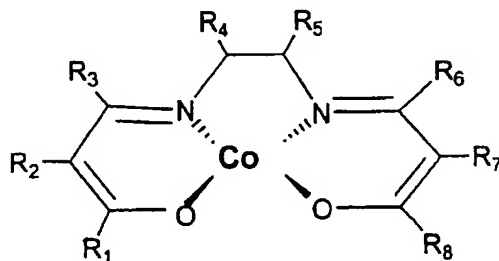
SUMMARY OF THE INVENTION

- 15 It is an object of the invention to provide novel cobalt compounds, including cobalt compounds containing targeting moities. It is a further object to provide methods for the inhibition of proteins, such as enzymes, using these cobalt complexes.

In accordance with these objects, compositions are provided comprising water soluble tetradentate Schiff's base complexes of Co(II).

Further provided are compounds having the structure comprising Formula 1:

Formula 1



In Formula 1, Co is either Co(II) or Co(III), and each of R₁, R₂, R₃, R₄, R₅, R₆, R₇, and R₈ is a hydrogen, an alkyl group, an aryl group, a hydrophobic organic acid, an alkyl alcohol, an alcohol, an alkyl amine group, an amine group, or a targeting moiety.

Also provided are compounds of Formula 1 wherein R₁, R₂, R₃ and R₄ are each hydrogen, alkyl, or aryl.

Also provided are protein-cobalt compound complexes comprising a protein attached to the cobalt compound of Formula 1.

Further provided are methods of inhibiting a selected protein comprising contacting the selected protein with the compound of Formula 1.

Additionally provided are methods of inhibiting zinc finger proteins comprising contacting a zinc finger protein with the cobalt compounds described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the inhibition of thrombin. 3.07×10^{-9} M thrombin at 25°C was assayed using Spectrozyme TH (American Diagnostics), and the reaction followed at 406 nm using a Hewlett Packard HP8452A diode array spectrophotometer with temperature control. All assays were performed in 10 mM Tris, 10 mM HEPES, 0.1% polyethylene glycol, 0.5 M NaCl, pH 7.8. The Co(III) carboxypropyl(NH_3)₂ (labeled as Co(carboxypropyl)) was coupled to the active site directed peptide NH_2 -GGGdFPR-CO- NH_2 (labeled as peptide dFPR). The observed inhibition greatly exceeded Co(carboxypropyl)(NH_3)₂, the peptide, and Co(III)acacen(NH_3)₂ Cl (labeled as Co(acacen)). This demonstrates the principle that coupling known inhibitors to the cobalt compound can greatly increase the potency of enzyme inhibition compared with the inhibitory activity of the uncoupled components.

Figure 2 depicts the structure of the Co(III)(acacen-GGGFPR)(NH_3)₂.

Figures 3A and 3B shows the temperature dependence of the enzyme inhibition rate correlates to the ligand exchange rate. (A): temperature dependence (see Example 3). (B): ligand exchange rate (see Example 3).

Figure 4 shows the inhibition of thrombin by [Co(III)(acacen)(NH_3)₂]Cl. Thrombin was incubated for 12 hours at room temperature with (A) 0 M Co(III) and B) 2.5 mM Co(III). Spectra were taken every 30 seconds for 30 minutes to monitor the release of p-nitroaniline by enzymatic hydrolysis of a commercial substrate.

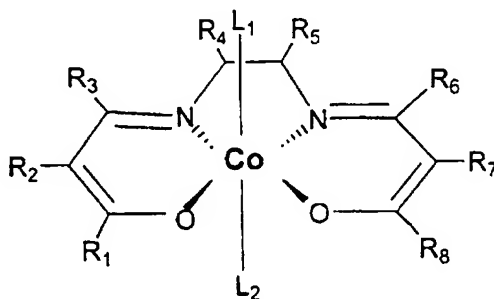
Figure 5 shows that inhibition of thrombin is dependent on the concentration of the inhibitor, the length of incubation, and the temperature of incubation.

Figures 6A, 6B, 6C, 6D, 6E, 6F, 6G, 6H, and 6I depict the structures of cobalt compounds of the invention that have been made. Unless noted, all of the compounds are Co(III).

DETAILED DESCRIPTION OF THE INVENTION

5 As is described below, the present invention is directed to cobalt compounds that can exchange or bind functional moieties such as histidine on a protein's surface resulting in the inactivation of a biological activity of the protein due to the complexing of the functional moiety to the cobalt compound.

10 The cobalt compounds of the invention utilize either Co(II) (also depicted herein as Co+2) or Co(III) (also depicted herein as Co+3). Generally, Co(II) compounds have up to four coordination atoms, and may contain a first axial ligand, although it is possible that water molecules may be weakly associated in one or both axial ligand positions. Similarly, Co(III) compounds have up to six coordination atoms, of which two are defined herein as axial ligand
15 positions. By "axial ligand" herein is meant a ligand L₁ or L₂ located at either the fifth or sixth coordination sites, generally depicted in the structure below:



Without being bound by theory, the cobalt compounds of the invention derive their biological activity by the substitution or addition of ligands in the axial positions. The biological activity of the cobalt compounds of the invention

- results from the binding of a new axial ligand, most preferably the nitrogen atom of imidazole of the side chain of histidine. Presumably the amino acid serving as a new axial ligand of the cobalt compound is required by the target protein for its biological activity. Thus, as is more fully described below,
- 5 proteins such as enzymes that utilize a histidine in the active site, or proteins that use histidine, for example, to bind essential metal ions, can be inactivated by the binding of the histidine in an axial ligand position of the cobalt compound, thus preventing the histidine from participating in its normal biological function.
- 10 When the cobalt is Co(III), the Co(III) complex is synthesized or formulated with two particular axial ligands, and then when the complex is added to a protein, for example, the original axial ligand or ligands are replaced by one or more ligands from a protein. This will occur either when the affinity of the protein axial ligand is higher for the cobalt compound as compared to the
- 15 original axial ligand, or when the new axial ligand is present in elevated concentrations such that the equilibrium of axial ligand binding favors the binding of the new axial ligand from the protein. Thus, Co(III) complexes are made with axial ligands that can be substituted with other ligands.
- Without being bound by theory, when the cobalt is Co(II), such complexes
- 20 may, under certain circumstances, have a first axial ligand. The Co(II) compounds of the invention are preferably synthesized with no axial ligands. Upon incubation with a protein, certain moieties, such as the nitrogen atom of the imidazole of the side chain of histidine, within the protein can become an axial ligand, resulting in a tightly-bound protein-cobalt compound complex.
- 25 This occurs when the Co(II) compound, with its four coordinating atoms from the Schiff's base, binds an imidazole moiety, for example, and is oxidized to a Co(III) compound. In one sense, this may be considered a redox reaction. since

the Co(II) compound is oxidized to a Co(III) compound upon binding to the protein. Thus, the imidazole axial ligand serves as a fifth coordinating atom, and is tightly bound.

5 In a preferred embodiment, the nitrogen atom of an imidazole side chain of the amino acid residue histidine, contained within a target protein, is the new axial ligand. While the examples and disclosure herein particularly describe this histidine embodiment, any "cobalt-reactive amino acid" may serve as the new axial ligand. A "cobalt-reactive amino acid" is one which is capable of binding to the cobalt compounds of the invention as an axial ligand. Thus, while the
10 nitrogen of the imidazole side chain of histidine is particularly preferred, alternative embodiments utilize the nitrogen atom of the aromatic indole side chain of tryptophan, the sulfur atoms of the side chains of cysteine and methionine, the amino groups of arginine, lysine, asparagine or glutamine as the moieties which may become axial ligands as outlined above. The
15 availability of these moieties may depend on the pH of the solution containing the protein or enzyme, since in the protonated state many of these moieties are not good electron donors suitable as axial ligands.

The present invention provides cobalt compounds that may be complexed with a protein containing a suitable new axial ligand.

20 In one embodiment, the present invention provides water-soluble tetradentate Schiff's base compounds of Co(II).

By the term "tetradentate" herein is meant that the Schiff's base compound, which is a ligand for the Co(II), has four coordinating atoms. In a preferred embodiment, there are two nitrogen atoms and two oxygen atoms which serve
25 as the coordinating atoms.

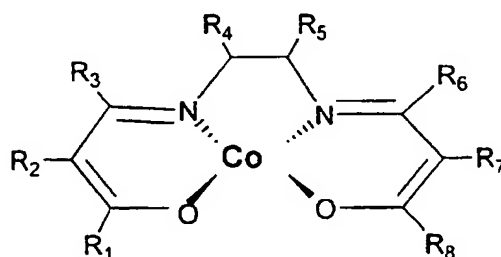
By the term "Schiff's base" herein is meant a substituted imine. The substituent groups are outlined below. Schiff's bases are generally the condensation products of amines and aliphatic aldehydes forming azomethines substituted on the nitrogen atom.

- 5 By the term "cobalt compound" herein is meant a tetradentate Schiff's base with a bound cobalt atom. The Schiff's base may be substituted or unsubstituted, and the cobalt may be Co(II) or Co(III).

In a preferred embodiment, the cobalt compounds have the structure depicted in Formula 1:

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Formula 1



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In this embodiment, Co is either Co(II) or Co(III). Each of R₁, R₂, R₃, R₄, R₅, R₆, R₇ and R₈ is a hydrogen, an alkyl group, an aryl group, a hydrophobic organic acid, an alkyl alcohol, an alcohol, an alkyl amine group, an amine group, or a targeting moiety. When Co is Co(II), at least one of R₁ through R₈ is hydrophilic such that the compound is soluble in aqueous solution. When Co is Co(III), at least one of R₁ through R₈ is a targeting moiety.

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By "alkyl" or "alkyl group" or grammatical equivalents herein is meant a straight or branched chain alkyl group, with straight chain alkyl groups being preferred. If branched, it may be branched at one or more positions, and unless specified, at any position. Also included within the definition of an alkyl group

are cycloalkyl groups such as C5 and C6 rings. In some cases, two R groups may be part of a ring structure, that is, they may be linked to form a cyclic structure.

5 The alkyl group may range from about 1 to 20 carbon atoms (C1 - C20), with a preferred embodiment utilizing from about 1 to about 10 carbon atoms (C1 - C10), with about C1 through about C5 being preferred. However, in some embodiments, the alkyl group may be larger, particularly if it is a straight chain alkyl. Particularly preferred is methyl.

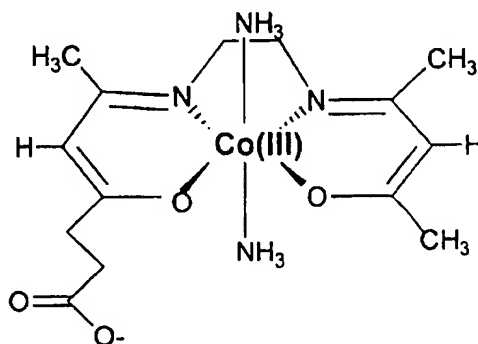
10 By "aryl" or "aryl group" herein is meant aromatic rings including phenyl, benzyl, and naphthyl, heterocyclic aromatic rings such as pyridine, furan, thiophene, pyrrole, indole and purine, and heterocyclic rings with nitrogen, oxygen, sulfur or phosphorus.

15 The alkyl and aryl groups may be substituted, for example, a phenyl group may be a substituted phenyl group. Suitable substitution groups include, but are not limited to, alkyl and aryl groups, halogens such as chlorine, bromine and fluorine, amines, carboxylic acids, and nitro groups.

20 By "hydrophilic organic acid" or grammatical equivalents herein is meant an alkyl group containing one or more carboxyl groups, -COOH, i.e. a carboxylic acid. As defined above, the alkyl group may be substituted or unsubstituted. C1 - C20 alkyl groups may be used with at least one carboxyl group attached to any one of the alkyl carbons, with C1 - C5 being preferred. In a preferred embodiment, the carboxyl group is attached to the terminal carbon of the alkyl group. Other preferred hydrophilic organic acids include phosphonates and sulfonates. A preferred hydrophilic organic acid is propionic acid.

In a preferred embodiment, only one of the R groups is a hydrophobic organic acid, since, in the case of Co(III), this may result in a compound that is neutrally charged, and thus may cross the blood-brain barrier. Particularly preferred is the structure depicted in Formula 2:

Formula 2



In addition, the length of the alkyl group shown in Formula 2 may be altered, either to encourage or prevent the carboxylic acid from "swinging around" to become an axial ligand.

By the term "amine" herein is meant an -NRR' group. In this embodiment, R and R' may be the same or different, and may be hydrogen, alkyl or aryl. A preferred -NRR' group is -NH₂.

By the term "alkyl amine group" herein is meant an alkyl group, as defined above, with a -NRR' group, as defined above. As defined above, the alkyl group may be substituted or unsubstituted. Preferred alkyl amine groups are n-propylamine and n-butylamine.

By the term "alkyl alcohol" herein is meant an alkyl group with an -OH group. As defined above, the alkyl group may be substituted or unsubstituted. The alkyl alcohol may be primary, secondary or tertiary, depending on the alkyl group. In a preferred embodiment, the alkyl alcohol is a straight chain primary

alkyl alcohol, generally containing at least 3 carbon atoms. Preferred alkyl alcohols include, but are not limited to, n-propyl alcohol, n-butyl alcohol, n-pentyl alcohol, n-heptyl alcohol, or n-octyl alcohol.

By the term "alcohol" herein is meant an -OH group.

- 5 In a preferred embodiment, at least one of R_1 - R_8 is a targeting moiety. It is preferred that only one of the R groups be a targeting moiety. In an alternative embodiment, more than one of the R groups may be a targeting moiety. When the Co of Formula 1 is Co(III), at least one of R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 and R_8 is a targeting moiety.
- 10 By the term "targeting moiety" herein is meant a functional group that will specifically interact with the target protein, and thus is used to target the cobalt compound to a particular target protein. That is, the cobalt compound is covalently linked to a targeting moiety that will specifically bind or associate with a target protein. For example, the cobalt compounds of the invention may
- 15 include a polypeptide inhibitor that is known to inhibit a protease, thus effectively increasing the local concentration of the cobalt compound at a functional site on the target protein. Suitable targeting moieties include, but are not limited to, polypeptides, nucleic acids, carbohydrates, lipids, hormones including proteinaceous and steroid hormones, growth factors, receptor ligands,
- 20 antigens and antibodies, and the like.

In a preferred embodiment, the cobalt compound containing a targeting moiety as one of the R groups inhibits a protein, which may or may not be an enzyme. By "inhibition of a protein" herein is meant that a biological activity of the protein is decreased or eliminated upon binding of the inhibitor. In the case of

25 enzymes, inhibition results in a decrease or loss of enzymatic activity. For

example, polypeptides comprising protease substrates or inhibitors are used as an R group on the cobalt compounds, to form cobalt compounds that will selectively inhibit the protease. Similarly, a cobalt compound containing an R group comprising a nucleic acid that specifically binds to a particular nucleic acid binding protein such as a transcription factor is used to selectively inhibit the transcription factor. These targeted cobalt compounds preferentially bind to the target site on the protein, favoring that site over non-specific binding to other sites or other proteins. This makes the resulting compound more effective at lower concentrations since fewer molecules interact at other sites and minimizes the side-effects due to inhibition of other proteins. Secondary interactions also increase the time spent at the target, giving more opportunity for ligand exchange.

In designing a cobalt compound for a particular protein, it is to be understood that the high affinity of the cobalt compound for an imidazole moiety, or the other possible reactive axial ligand moieties, is such that the cobalt compound need not be a perfect fit in the active site. Rather, what is important is that the cobalt compound be able to approach the target axial ligand moiety. For targeting active site residues of enzymes, for example, the cobalt compounds should generally not be larger than typical enzyme substrates or inhibitors. The gross structure and surface properties of the cobalt compound reagent will determine its outer sphere interaction with the desired biological active site. Specificity in outer sphere interactions is optimized by variations in size, charge, flexibility, stereochemistry, and surface properties of the cobalt compound reagent. Thus, in designing an appropriate inhibitor, the characteristics of the protein or enzyme target are exploited. In addition, as is shown in the Examples, increasing the local concentration of the cobalt compound at or near the active site of the protein is sufficient to increase the binding of the cobalt compound and thus the inhibition of the biological

activity of the protein, effectively decreasing the K_m or K_i values, in the case of enzymatic inhibition.

When the target protein is known to have a histidine or other cobalt reactive amino acid that is functionally important, either Co(II) or Co(III) may be used, with Co(III) being preferred. When the functional mechanism of the target protein is unknown, either Co(II) or Co(III) may be used, with Co(II) being preferred. In a preferred embodiment, the cobalt reactive amino acid is also close to the binding pocket or site of the targeting moiety.

By the term "polypeptide" herein is meant a compound ranging from about 2 to about 15 amino acid residues covalently linked by peptide bonds. Preferred embodiments utilize polypeptides from about 2 to about 8 amino acids, with about 4 to about 6 being the most preferred. Preferably, the amino acids are naturally occurring amino acids in the L-configuration, although amino acid analogs are also useful, as outlined below. Under certain circumstances, the polypeptide may be only a single amino acid residue. Additionally, in some embodiments, the polypeptide may be larger, and may even be a protein, although this is not preferred. In one embodiment, the polypeptide is glycosylated.

Also included within the definition of polypeptide are peptidomimetic structures or amino acid analogs. Thus, for example, non-naturally occurring side chains or linkages may be used, for example to prevent or retard *in vivo* degradations. Alternatively, the amino acid side chains may be in the (R) or D-configuration. Additionally, the amino acids, normally linked via a peptide bond or linkage, i.e. a peptidic carbamoyl group, i.e. -CONH-, may be linked via peptidomimetic bonds. These peptidomimetic bonds include CH_2-NH- , $CO-CH_2$, azapeptide and retroinversion bonds.

As used herein, "nucleic acid" may refer to either DNA or RNA, or molecules which contain both deoxy- and ribonucleotides. Generally, the nucleic acid is an oligonucleotide, ranging from about 3 nucleotides to about 50 nucleotides, with from about 12 to about 36 being particularly preferred, and at least 21 nucleotides being especially preferred. When the nucleic acid is used solely to confer solubility, the nucleic acid may be smaller, and in some embodiments may be a single nucleotide. The nucleotides may be naturally occurring nucleotides, or synthetic nucleotides, and may be any combination of natural and synthetic nucleotides, although uracil, adenine, thymine, cytosine, guanine, and inosine are preferred. As is more fully described below, the nucleic acids include genomic DNA, cDNA and oligonucleotides including sense and anti-sense nucleic acids. The nucleic acid may be double stranded, single stranded, or contain portions of both double stranded or single stranded sequence. In a preferred embodiment, for example when the nucleic acid is used to target a zinc finger transcription factor, the nucleic acid is double stranded, as zinc fingers bind to the major groove of double stranded nucleic acids.

A nucleic acid will generally contain phosphodiester bonds, although in some cases, as outlined below, a nucleic acid may have an analogous backbone, comprising, for example, phosphoramidate (Beaucage et al., Tetrahedron 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 (1986)), phosphorothioate, phosphorodithioate, O-methylphosphoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), or peptide nucleic acid linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al.,

Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993)). These modifications of the ribose phosphate backbone may be done to facilitate the addition of cobalt compounds, as outlined below, or to increase the stability and half-life of such molecules in physiological environments.

5 By "carbohydrate" herein is meant a compound with the general formula $C_x(H_2O)_y$. Monosaccharides, disaccharides, and oligo- or polysaccharides are all included within the definition and comprise polymers of various sugar molecules linked via glycosidic linkages. Particularly preferred carbohydrates are those that comprise all or part of the carbohydrate component of
10 glycosylated proteins, including monomers and oligomers of galactose, mannose, fucose, galactosamine, (particularly N-acetylglucosamine), glucosamine, glucose and sialic acid, and in particular the glycosylation component that allows binding to certain receptors such as cell surface receptors. Other carbohydrates comprise monomers and polymers of glucose,
15 ribose, lactose, raffinose, fructose, and other biologically significant carbohydrates.

"Lipid" as used herein includes fats, fatty oils, waxes, phospholipids, glycolipids, terpenes, fatty acids, and glycerides, particularly the triglycerides. Also included within the definition of lipids are the eicosanoids, steroids and
20 sterols, some of which are also hormones, such as prostaglandins, opiates, and cholesterol. Hormones include both steroid hormones and proteinaceous hormones, including, but not limited to, epinephrine, thyroxine, oxytocin, insulin, thyroid-stimulating hormone, calcitonin, chorionic gonadotropin, corticotropin, follicle-stimulating hormone, glucagon, leuteinizing hormone,
25 lipotropin, melanocyte-stimulating hormone, norepinephrine, parathryroid hormone, vasopressin, enkephalins, serotonin, estradiol, progesterone, testosterone, cortisone, and glucocorticoids. Receptor ligands include ligands

that bind to receptors such as cell surface receptors, which include hormones, lipids, proteins, glycoproteins, signal transducers, growth factors, cytokines, and others.

5 In one embodiment, the targeting moiety is chosen just to confer solubility on the Co(II) or cobalt compound. Thus, for example, the actual sequence of amino acid residues or nucleotides is not critical. Alternatively, as outlined above, the targeting moiety is chosen to target a particular protein or enzyme, and thus, when the targeting moiety is a polypeptide for example, the actual sequence of amino acids is important.

10 In a preferred embodiment, at least one of R_1 - R_8 of Formula 1 is a polypeptide. In this embodiment, the polypeptide is chosen on the basis of the target protein or enzyme to be inhibited.

15 For example, when the target enzyme is a protease, the polypeptide will mimic or comprise an enzyme substrate or the reactive site of an inhibitor. When the polypeptide comprises an inhibitor, the inhibitor may be either a reversible or irreversible inhibitor. The sequence of the polypeptide is chosen to allow the binding of the polypeptide to the active site of the protease.

20 The polypeptide and the site of attachment of the polypeptide to the cobalt compound, will be chosen to maximize the interaction of the cobalt with the active site histidine. That is, as is explained below, the polypeptide may be attached to the cobalt compound at the N-terminal end, the C-terminal end, or internally, via one or more amino acid side chains.

As is well known in the art, the active site histidine of many enzymes is close to the S1-S1' position of the enzyme's substrate (or inhibitor) binding site.

Examples include the serine and cysteine proteases. Thus, in a preferred embodiment, the polypeptide is chosen to allow optimum interaction of the cobalt compound with the active site histidine. For example, the polypeptide may comprise roughly the P4 through P1 residues of a substrate or inhibitor (which occupy the S4 to S1 positions of the enzyme's binding site), and be attached at the C-terminal end (P1) to the cobalt compound, to maximize the steric interaction of the cobalt compound with the active site of the enzyme, and particularly the active site histidine. Alternatively, the polypeptide may comprise the P1' through P4' residues (corresponding to the S1' to S4' positions), with attachment at the N-terminus (P1'). In a further embodiment, the polypeptide spans the P1-P1' site, but has an internal attachment at or near the P1 or P1' residues, to similarly maximize the interaction of the cobalt compound with the active site histidine. These types of attachments are described below. However, as noted above, the interaction need not be perfect to allow inhibition, since it appears that increasing the local concentration of the cobalt compound near the active site is sufficient.

Thus, the present invention allows a known enzymatic substrate to be used as an inhibitor, as well as increasing the efficiency of known inhibitors, for example via decreasing the K_i . A wide variety of enzyme substrates and inhibitors for a variety of proteases containing either an active site histidine or an essential metal ion coordinated by a histidine are known in the art. In addition, the morphological properties of enzymes for which the crystal structures are known are used to design appropriate cobalt compounds. Alternative embodiments utilize known characteristics about surface charge and hydrophobicity, and substrate and inhibitor specificity.

In a preferred embodiment, the K_i of the polypeptide inhibitor is decreased as a result of attachment to the cobalt compound. That is, the inhibitor becomes a

better inhibitor as a result of the attachment of the cobalt compound. Thus, the cobalt compound is effective at lower concentrations since fewer molecules are wasted at other sites.

5 In a preferred embodiment, at least one of the R groups is a nucleic acid used to target the cobalt compound to a particular protein or enzyme. For example, the target protein can be a nucleic acid binding protein that has at least one histidine that is important in biological activity, such as a zinc finger protein.

10 As is known for zinc finger proteins that bind nucleic acids, it appears that each zinc finger interacts or binds to three base pairs of nucleic acid (see Berg, supra). Thus, the actual sequence of the nucleic acid used to target a nucleic acid binding protein will vary depending on the target protein. Nucleic acid sequences and their target binding proteins are known in the art.

15 As with the polypeptides, the cobalt compound can be attached to the nucleic acid in a variety of ways in a variety of positions; the actual methods are described below. The attachment site is chosen to maximize the interaction of a cobalt-reactive amino acid such as histidine that is essential for metal ion binding (or an active site histidine) with the cobalt compound. In a preferred embodiment, the backbone of the nucleic acid is modified to contain a functional group that can be used for attachment to the cobalt compound. This functional group may be added to either the 5' or 3' end of the nucleic acid, or to an internal nucleotide. For example, the nucleic acid may be synthesized to contain amino-modified nucleotides using techniques well known in the art (see for example Imazawa et al., J. Org. Chem. 44:2039-2041 (1979); Miller et al., Nucleosides, Nucleotides 12:785-792 (1993); and WO95/15971, and references cited therein). In this embodiment, amine groups are added to the ribophosphate backbone at the 2' or 3' position, thus allowing the attachment of

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the nucleic acid to the cobalt at either the 5' or 3' end, or to an internal nucleotide. These amine groups are then used to couple the nucleic acid to the cobalt compound. Alternatively, nucleotide dimers, containing phosphoramidate, phosphorothioate, phosphorodithioate, or O-methylphosphoroamidite linkages may be made, and added to the nucleic acid at any position during synthesis, and the nitrogen or sulfur atom used for attachment using well known techniques, as outlined below. Additionally, the phosphorus atom of the backbone may be used, or linkers, as is known in the art (see for example Thuong et al., *Angew. Chem. Intl. Ed. Engl.* 32:666-690 (1993); and Mergny et al., *Nucleic Acid Res.* 22:920-928 (1994)).

When Co is Co(II) in Formula 1, at least one of R₁, R₂, R₃, R₄, R₅, R₆, R₇ and R₈ is hydrophilic such that the Co(II) compound is soluble in aqueous solution. In one embodiment, only one of the R groups is hydrophilic and the other R groups are chosen such that the single hydrophilic R group is sufficient to confer water solubility to the Co(II) compound. In a preferred embodiment, R₁ is hydrophilic, for example, n-propyl alcohol. In alternative embodiments, two, three, four, five, six, seven or eight R groups are hydrophilic. In a preferred embodiment, the hydrophilic group is a targeting moiety, and preferably either a polypeptide or a nucleic acid.

By "soluble in aqueous solution" herein is meant that the Co(II) compound has appreciable solubility in aqueous solution and other physiological buffers and solutions. Solubility may be measured in a variety of ways. In one embodiment, solubility is measured using the United States Pharmacopeia solubility classifications, with the Co(II) compound being either very soluble (requiring less than one part of solvent for 1 part of solute), freely soluble (requiring one to ten parts solvent per 1 part solute), soluble (requiring ten to thirty parts solvent per 1 part solute), sparingly soluble (requiring 30 to 100

parts solvent per 1 part solute), or slightly soluble (requiring 100 -1000 parts solvent per 1 part solute). Alternatively, since cobalt containing compounds are generally colored, the appearance of a color upon addition to a colorless aqueous solution indicates an acceptable level of solubility. For example,
5 many Co(II) Schiff's base complexes have a yellow or orange color.

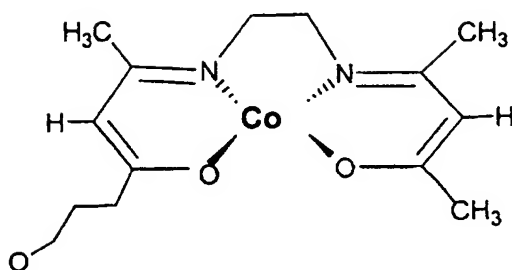
Testing whether a particular Co(II) compound is soluble in aqueous solution is routine, as will be appreciated by those in the art. For example, as noted above, the appearance of a Schiff's base Co(II) complex color upon addition to a colorless aqueous solution indicates solubility. Alternatively, the parts of
10 solvent required to solubilize a single part of Co(II) compound may be measured, or solubility in gm/ml may be determined.

In a preferred embodiment, the cobalt compounds depicted in Formula 1 have a regiospecific hydrophilicity. That is, R_1 , R_2 , R_3 , and R_4 , are either hydrogen, alkyl or aryl, and are therefore hydrophobic, and at least one of R_5 , R_6 , R_7 and
15 R_8 is hydrophilic. However, other combinations resulting in amphiphathic characteristics are also possible, as will be appreciated by those in the art. This is particularly desirable since this regiospecific hydrophilicity allows better positioning of the cobalt compound in or near the active site or on the surface of a protein or enzyme, as is discussed below. Without being bound by theory,
20 it appears that this regiospecific hydrophilicity/hydrophobicity allows the cobalt compound to more efficiently interact with the protein or enzyme, which generally displays both hydrophobic and hydrophilic regions.

Particularly preferred embodiments of the present invention include the structure depicted in Formula 4, wherein R_1 is n-propyl alcohol, R_2 is
25 hydrogen, R_3 is methyl, R_6 is methyl, R_7 is hydrogen, R_8 is methyl, and R_4 and R_5 are hydrogen:

-21-

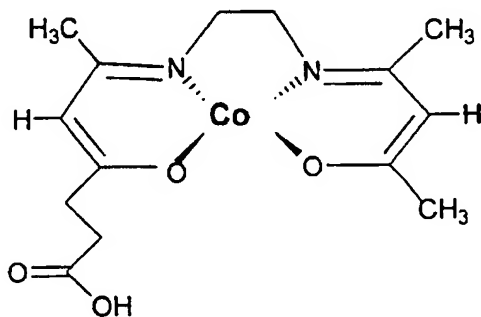
Formula 4



In this embodiment, the Co can be either Co(II) or Co(III).

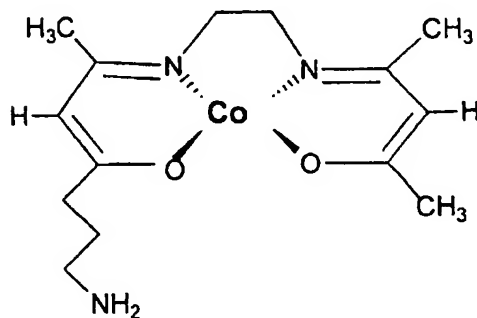
The structures depicted in Formulas 5 and 6 are also preferred:

Formula 5



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Formula 6



In a preferred embodiment, the cobalt complexes may have groups that alter the redox potential, oxidation stability, or ability of the compound to exchange an axial ligand. For example, many of the Co(II) complexes of the invention are

sensitive to air oxidation. That is, in the presence of atmospheric oxygen, they may be oxidized. Thus, in a preferred embodiment, the Co(II) complexes are synthesized and utilized in the absence of air.

Thus, in a further embodiment, the Co complexes are additionally modified to make them air stable compounds. For example, replacement of a methyl R group in a complex of the invention with a trifluoromethyl group results in a positive shift of the metal oxidation potential, stabilizing the metal complex with respect to air oxidation. For example, 1,1,1-trifluoro-2,4-pentanedione is commercially available, and may be used to synthesize trifluoromethyl derivatives of the Co(II) complexes disclosed herein. Further well known modifications such as chlorination of the Schiff's base macrocycle also greatly enhance the stability of these complexes with respect to air oxidation. Thus, the use of trifluoromethyl groups alone or in conjunction with chlorination of the macrocycle will result in a soluble air stable Co(II) macrocycle complex.

These types of derivatives may also be made to adjust the redox potential of complexes to modulate their reactivity with other compounds.

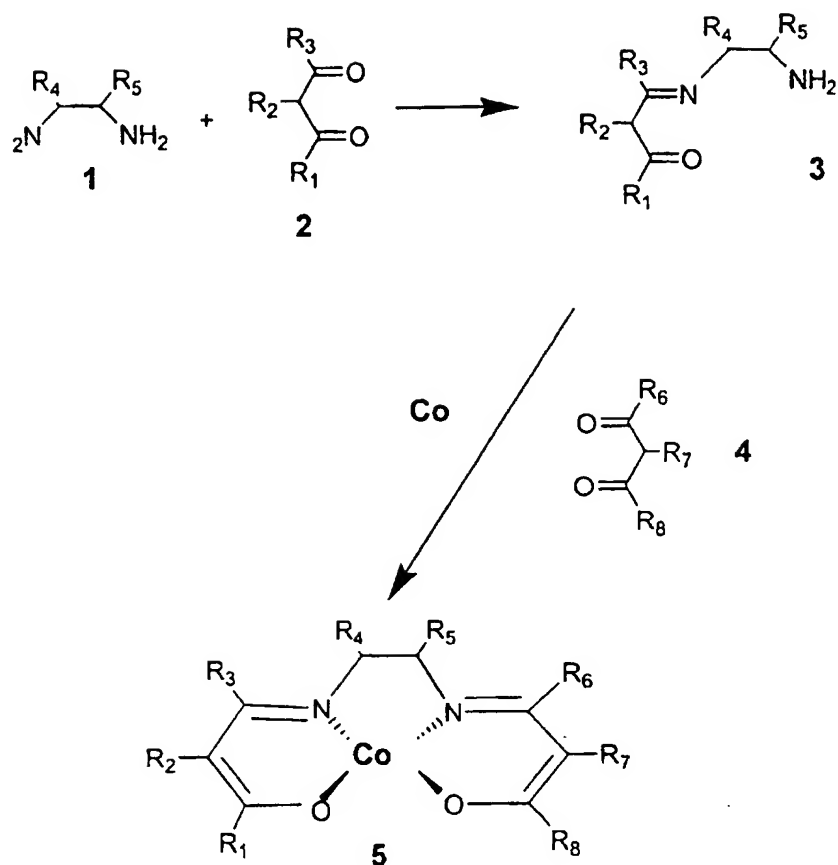
Similarly, the addition of electron donating or electron withdrawing groups may effect the activity of the cobalt compound with respect to the ability to exchange an axial ligand. As shown in the examples, the addition of trifluoromethyl R groups at the R₁ and R₈ positions basically eliminates the reactivity of the Co(III) compound towards new axial ligands. Electron withdrawing or donating groups are preferably added at the R₁ and/or R₈ positions, as this is easiest for synthesis, as well as the preferred position for electronic coupling. The R₂ and/or R₇ positions are also preferred. It is also possible to put electron donating groups at the R₃ and R₆ positions, but if R₃ and/or R₆ contain an electron withdrawing group then the compound may be

difficult to synthesize using the schemes depicted herein. Suitable electron withdrawing groups include, but are not limited to, halides (F, Cl, Br, I, in decreasing order of electron withdrawing strength), phenyl and substituted phenyl groups such as nitro-phenyl, amines and quaternary amines, thiols, nitro groups, carboxy groups, nitrile, alkynes and alkanes, sulfonyls, and others known in the art. Suitable electron donating groups include, but are not limited to, -OCH₃, methyl, carboxylate, and ether.

Once the R groups are chosen, the preparation of the cobalt compounds of the invention proceeds as outlined below.

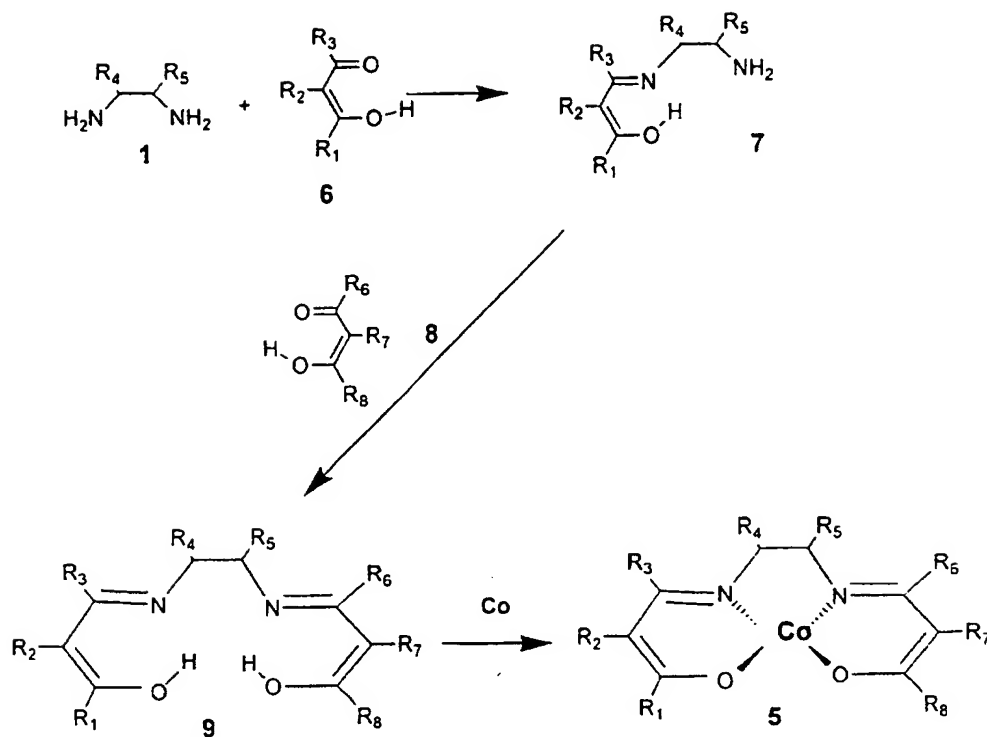
10 Generally, the cobalt compounds of the invention are synthesized as generically disclosed below in Scheme I, using the general methods of Costa et al., J. Organometal. Chem., 6:181 (1966), which describes the preparation of derivatives of the components used to make the ligands used in the invention, such as acetylacetonate ethylenediamine (acacen).

Scheme I



Compounds 1 (ethylenediamine, "en"), 2 and 4 are generally made using techniques well known in the art. Compounds 2 and 4 are aliphatic β -diketones, and compound 2 is an aliphatic amine. It will be understood by those skilled in the art that compounds 2, 3 and 4 are the resonance structures of compounds 6, 7, and 8 shown below in Scheme II. Compounds 6 and 8 are acetylacetonone derivatives ("acac"), and compound 7 is the "monoacacen" product.

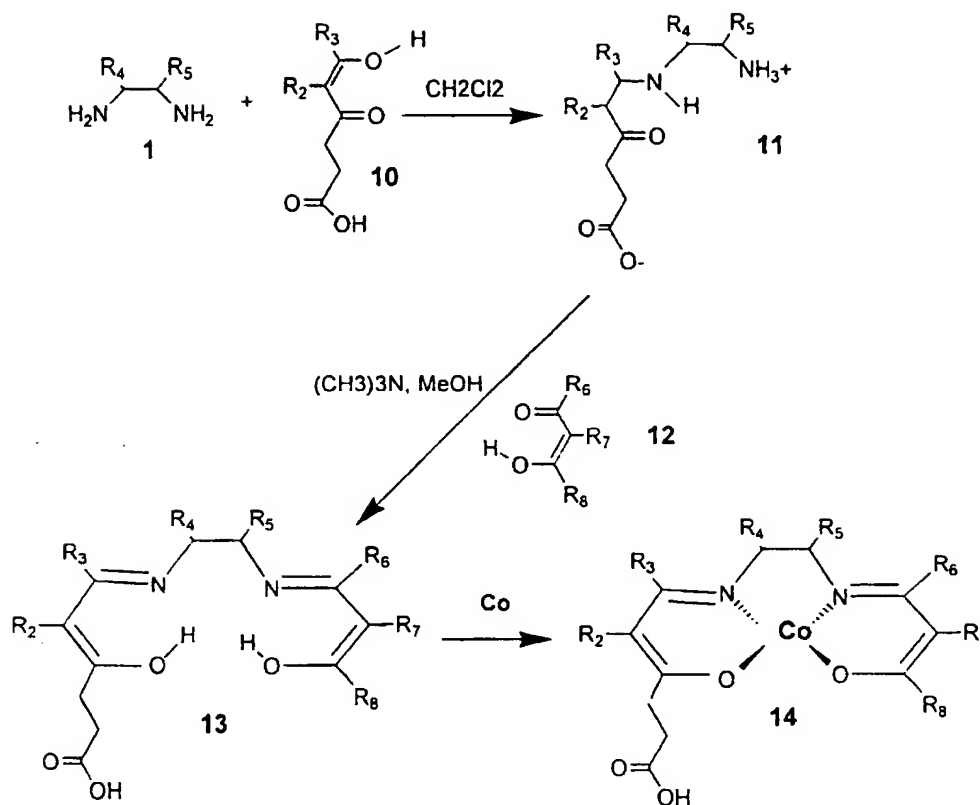
Scheme II



When the Co is Co(III), the axial ligands are usually added in the last step.

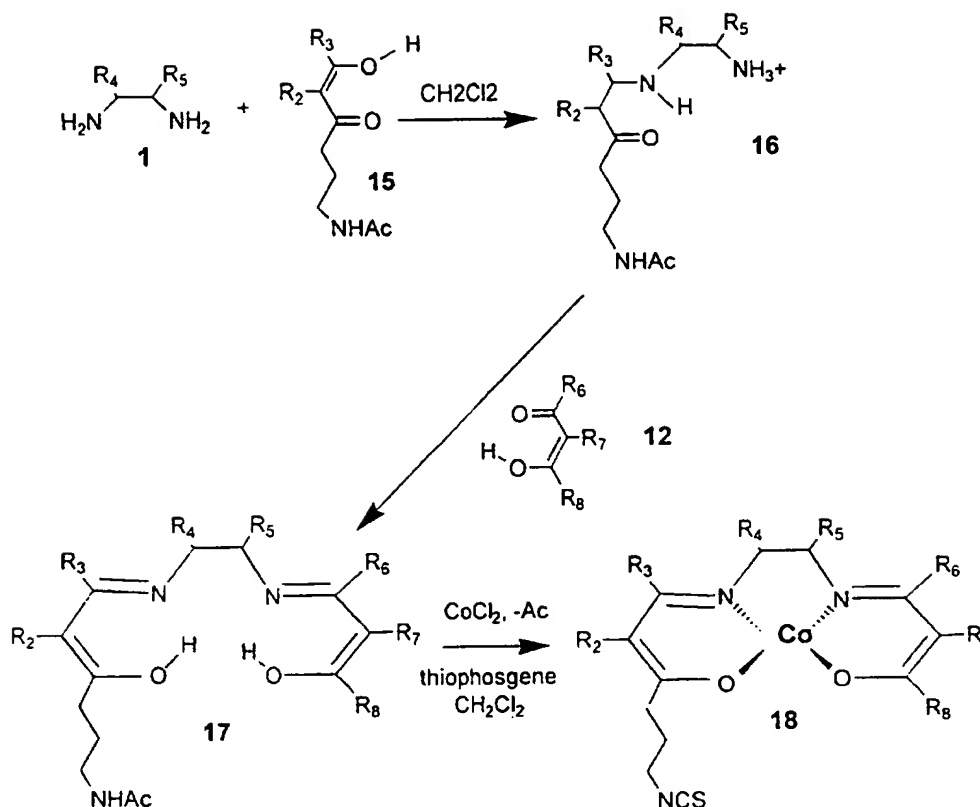
Of particular use for attachment of targeting moieties and particularly polypeptide and nucleic acid R groups are cobalt compounds with carboxy and amino groups. Cobalt compounds utilizing carboxylic acids are synthesized as depicted below in Scheme III:

Scheme III



The amino-derivative of the core cobalt compound is synthesized as follows:

Scheme IV



The NCS group may then be used for coupling, as is known in the art.

In the case where the R group is a targeting moiety, the cobalt compounds are generally constructed in three phases. First, the core cobalt compound is synthesized with a functional moiety that can be used to couple the targeting moiety. For example, the core cobalt compound is made with an amine, a carboxy or a sulfhydryl group. Next, the R group, comprising a targeting moiety, is made, which also contains a functional moiety that can be used for attachment. In some instances, other reactive groups of the targeting moiety

are protected to prevent them from reacting with the functional group of the core cobalt compound. For example, amino acid side chains containing amino groups, such as arginine, may need to be protected to prevent the side chain from reacting, although in some embodiments the attachment is done via a functional moiety of an amino acid side chain. Protecting groups and techniques are well known in the art. Once the core cobalt compound and the R group are made, they can be attached by reacting the functional groups. In some instances, the linkage is direct; for example a cobalt compound containing a carboxy R group may be directly linked to an amino terminus of a polypeptide, as is depicted in the Examples. C-terminal attachment may be done using a cobalt compound with a amino functional moiety. As is known in the art, this direct linkage may be done in organic solvents or alternatively using coupling reagents such as 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (see generally, March, *Advanced Organic Chemistry*, 3rd Ed. Kiley & Sons, Inc. (1985) ; see also the 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference).

In a preferred embodiment, the linkage between the two functional moieties may utilize a linker, also well known in the art. For example, two amino groups may be linked via a stable bifunctional groups as are well known in the art, including homobifunctional and heterobifunctional linkers (see Pierce Catalog and Handbook, pages 155-200). In an additional embodiment, carboxy groups (either from the polymer or from the cell targeting moiety) may be derivatized using well known linkers (see the Pierce catalog). For example, carbodiimides activate carboxy groups for attack by good nucleophiles such as amines (see Torchilin et al., *Critical Rev. Therapeutic Drug Carrier Systems*, 7(4):275-308 (1991), expressly incorporated herein). Sulfhydryl groups may be

added to amines or carboxy groups with heterobifunctional linkers (see the Pierce catalog).

It should be understood that the attachment may be done in a variety of ways, including those listed above. What is important is that manner of attachment
5 does not significantly alter the functionality of the targeting moiety; that is, they are still able to bind to the target protein. As will be appreciated by those in the art, this is easily verified.

As will be appreciated in the art, a number of functional groups of the targeting moiety may be used for covalent coupling, such as alcohols, amino groups, and
10 carboxy groups. Alternatively, the targeting moiety may be derivatized to contain a functional moiety, such as through the addition of a linker containing a functional moiety. When a polypeptide is to be used as an R group, a preferred embodiment utilizes an amino group of the polypeptide. The N-terminal amino group may be used, or alternatively, an amino group of an
15 amino acid side chain, such as the amine groups of arginine, asparagine, glutamine, lysine, histidine and tryptophan. Similarly, the linkage may be accomplished using the sulfur atoms of the side chains of methionine or cysteine. The carboxy groups of the side chains of glutamic acid and aspartic acid may also be used.

20 When the R group is a nucleic acid, a variety of positions may be used as the site of covalent attachment to the cobalt compound. In a preferred embodiment, the ribophosphate backbone of the nucleic acid is modified to contain a functional moiety (see for example Meade et al., *Angewandte Chemie*, English Edition, 34(3):352-354 (1995), and references cited therein;
25 Imazawa et al., *supra*, Miller et al., *supra*). For example, in a preferred embodiment, an amino group is added at the 2' or 3' position of the sugar using

techniques well known in the art. In one embodiment, this is done by adding additional nucleotides that have an added amino group to the nucleic acid; that is, as shown in the Examples, one or more "extra" nucleotides is added to the targeting nucleic acid. Alternatively, the phosphodiester linkage between two nucleotides may be altered to form phosphoramidate, phosphorothioate, phosphorodithioate, or O-methylphosphoroamidite linkages, as is known in the art. The nitrogen or sulfur atoms are then used as functional moieties. The nucleotide dimer, containing the altered linkage, may be added to the nucleotide at any position. Functional groups on the nucleotide bases themselves may also be used, such as the amino groups on adenosine and cytosine, or modified bases such as is known for thymine (see for example Telser et al., J. Amer. Chem. Soc. 111:7221-7226 (1991); Unglisch et al., Angew. Chem 103:629-646 (1991); Angew. Chem. Int. Ed. Engl. 30:613-629 (1991); Goodchild, Bioconjugate Chem. 1:165-187 (1990); and Brun et al., J. Amer. Chem. Soc. 113:8153-8159 (1991)). Then the nucleic acid containing the functional group may be added to the cobalt compound either directly or via a linker, as is outlined above for polypeptides.

Similarly, other targeting moieties such as carbohydrate, lipid, and hormone targeting moieties may be altered to contain functional groups for linkages, as will be appreciated in the art, or derivatized with linkers containing functional groups. As discussed above, the functional group for coupling should not prevent the binding of the targeting moiety to the target protein, and preferably does not affect the binding. Generally, these targeting moieties containing suitable functional groups are made using well known techniques.

Once synthesized, the cobalt compounds of the invention find use in a number of applications. At the broadest level, the Co(II) compounds are useful as reducing agents in aqueous solution.

5 In one embodiment, the cobalt compounds of the invention are useful as general bacteriostatic or bactericidal agents, antimicrobial agents and/or antiviral agents, for both topical and other therapeutic applications. For example, topical antimicrobial agents may be useful in cleaning and disinfectant compositions, as will be appreciated in the art. Therapeutic uses of antimicrobial and antiviral agents are also well known.

10 The compounds are assayed for antiviral, antimicrobial and antibacterial activity using techniques well known in the art; for example, bactericidal activity may be measured using the techniques outlined in example VI of U.S. Patent 5,049,557. Both *in vitro* and *in vivo* antiviral activity may be measured using the techniques outlined in U.S. Patent No. 5,210,096.

15 The cobalt compounds of the invention can also be used to label proteins. The Co(II) compounds of the invention are preferably made with no axial ligands, and the Co(III) compounds are generally made with two axial ligands. Upon incubation with a protein, certain moieties on the protein will become axial ligands, resulting in a tightly bound protein-cobalt compound complex. Since
20 cobalt-containing compounds may be detected spectrophotometrically, the result is a labeled protein. The preferred axial ligand from a protein is the imidazole side chain of histidine. Thus, a protein with one or more histidine residues either at the surface of the protein or otherwise accessible to the solvent can be labeled using the cobalt compounds of the invention.

In this embodiment, the cobalt compounds of the invention are added or contacted with the target protein. The excess cobalt compound may be separated, and the labeled protein, with the attached Co(III) compound, is detected spectrophotometrically. The Co(III) compounds are generally
5 detected at 280, 338, and 451 nm, although a broad range from 280 to 500 nm may be useful.

The stoichiometry of the bound cobalt compound to protein will vary depending on the number of potential axial ligands in or at the active site or on the surface of the protein, and may be determined spectrophotometrically, as is
10 understood in the art. Thus, for example, a protein which has four accessible histidines will generally bind four cobalt compounds, etc.

Thus, the cobalt compounds of the present invention are also useful in probing the surface characteristics of a protein.

When used to bind or label proteins, the cobalt compounds can be coupled,
15 using standard technology, to affinity chromatography columns. These columns may then be used to separate proteins from a sample. For example, depending on the specificity of the cobalt complex, proteins may be removed from a sample, or specific proteins, such as those containing histidines at or near the active site may be separated from other components of the sample.

20 In a preferred embodiment, the cobalt compounds are useful as enzyme inhibitors. The mechanism of inactivation is similar to the mechanism of protein labeling. In this embodiment, an enzyme has one or more moieties capable of binding in an axial position in the cobalt compounds of the invention. One or more of such moieties are also functionally important for

enzymatic activity, and are inactivated upon contact with the cobalt compounds of the invention.

For example, enzymes which have histidine as an active site catalytic residue or have histidines which are functionally important for enzymatic activity are particularly preferred. Enzymes such as the serine proteases (trypsin, subtilisin, 5 chymotrypsin, elastase, thrombin, factor Xa, lysozyme, and others known in the art), cysteine proteases such as the cathepsins and interleukin converting enzyme; RNase H, thermolysin and lactate dehydrogenase all have active site histidines and thus may be inhibited with the compounds of the present 10 invention.

In this embodiment, a cobalt compound is contacted with the target enzyme. The imidazole side chain of an active site histidine binds to the cobalt compound as an axial ligand. In the case of Co(II), this occurs with a simultaneous or rapid oxidation of the Co(II) compound to form an enzyme- 15 Co(III) compound complex. This is termed "redox coupling".

The binding (and oxidation, in the case of the Co(II) compound) results in the inhibition of the enzyme. The exact mechanism of the inactivation is unknown; however, several possibilities exist. The bound cobalt compound, which after binding and oxidation is a Co(III) compound, may sterically 20 interfere with catalytic activity, i.e. it may be bound in or near the catalytic active site. Alternatively, the bound cobalt compound may interfere with the catalytic mechanism, i.e. by binding to a catalytic histidine. Additionally, in the case of Co(II), it is also possible that a functionally important moiety at the active site is reduced by the Co(II) compound, and thus the enzyme is 25 inactivated.

In a preferred embodiment, the inactivation of the enzyme by the cobalt compound inhibitor is effectively irreversible.

In alternative embodiments, the reactive axial ligand from the enzyme is the indole side chain of tryptophan or the side chains of cysteine, methionine, arginine, lysine, asparagine, glutamine, aspartate or glutamate. As outlined above, the availability of these moieties may depend on the pH of the solution containing the protein or enzyme, since in the protonated state these moieties are not good electron donors suitable as axial ligands. Thus, enzymes with these groups within the active site, or enzymes which have functionally important tryptophans, cysteines, or methionines may be inactivated by the cobalt compounds of the present invention, as outlined above.

In an additional embodiment, metalloproteins are inactivated with the cobalt compounds of the present invention. Generally, the metals of metalloproteins have ligands such as histidine, cysteine and methionine. If one or more of these residues are inactivated using these cobalt compounds, the binding of the metal atom may be decreased or eliminated, thus reducing or eliminating biological activity. Particular metalloproteins include, but are not limited to, nucleic acid binding proteins such as "zinc finger" proteins and hemerythrin. Zinc finger proteins utilize histidine and cysteine to bind zinc ions (see Berg, Ann. Rev. Biophys. Biophys. Chem 19:405-421 (1990), Berg, Science 232:485 (1986), and Berg, Prog. Inorg. Chem. 37:143 (1989), hereby expressly incorporated by reference). Zinc finger proteins have been shown to bind nucleic acids and thus play a role in a variety of gene regulatory processes. Zinc finger proteins include transcription factors and other nucleic acid-binding and gene-regulatory proteins (see Berg, Science, supra), and are found in eukaryotes, prokaryotes, and viruses. Other zinc finger proteins suitable for inactivation by the compounds of the present invention include the nucleic acid

binding domain of steroid and thyroid hormone receptors and the human oncogene product GLI (see Pavlitch et al., Science 261:1701 (1993); Kinzler et al., Nature 332:371 (1988), that contains five zinc finger domains. In a preferred embodiment, one or more of the zinc finger domains utilizes at least one histidine to bind zinc, with the proteins that utilize two histidines being preferred. In some cases the metal is bound exclusively by cysteines.

When the metalloprotein is a metalloenzyme, displacement of the active site metal by the cobalt complex may modulate enzyme activity. Such metalloenzymes include, but are not limited to, the carboxypeptidases, carbonic anhydrase, thermolysin, collagenase, histidinol dehydrogenase, leukotriene A4 hydrolase, adenosine deaminase, superoxide dismutase, alcohol dehydrogenase, lactate dehydrogenase, stromalysin, aminoacylase, tryptophanyl-tRNA synthetase, and others known in the art.

In a preferred embodiment, serine and cysteine proteases are inhibited.

In a preferred embodiment, the enzyme to be inhibited is carbonic anhydrase. Carbonic anhydrase has been implicated in diabetes, ocular disease such as glaucoma, and seizures and convulsions. Accordingly, inhibitors of carbonic anhydrase, such as the Co(II) complexes of the present invention, are useful in the treatment of these conditions.

Thus, in one embodiment, the Co(II) complexes are useful in the treatment of elevated intraocular pressure and glaucoma. Carbonic anhydrase has been implicated in elevated intraocular pressure, and carbonic anhydrase inhibitors have been shown to be efficacious in decreasing this pressure in animals and humans (see Sharir et al., Experimental Eye Res. 58(1):107-116 (1994):

Rassam et al., Eye 7(Pt 5):697-702 (1993); Gunning et al., Graefes Archive for Clinical and Experimental Ophthalmology 231(7):384 (1993)).

5 In an additional embodiment, the Co(II) compounds are useful in the treatment of seizures and convulsions. Carbonic anhydrase II deficient mice have been shown to have increased resistance to chemically induced seizures, and pretreatment with carbonic anhydrase inhibitors has been shown to increase the resistance of normal mice to chemically induced seizures. See Velisek et al., Epilepsy Res. 14(2):115-121 (1993).

10 In a further embodiment, the Co(II) compounds are useful in the treatment of diabetes and abnormal renal function. Elevated levels of carbonic anhydrase have been associated with metabolic diseases like diabetes mellitus and hypertension, and carbonic anhydrase inhibitors have been suggested for treatment. See Parui et al., Biochem. International 26(5):809-820 (1992); Parui et al, Biochem. International 23(4):779-89 (1991); Dodgson et al., Arch.
15 Biochem. Biophys. 277(2):410-4 (1990); Hannedouche et al., Clinical Sci. 81(4):457-64 (1991).

In a preferred embodiment, the cobalt compounds find use in the inhibition of proteins and enzymes of tumor cells. As outlined above, Co(III) "acacen" compounds can exchange an axial ligand for a different one by a dissociative
20 mechanism with the slow loss of one axial ligand to form a five coordinate intermediate, followed by binding to another suitable ligand. For most cobalt complexes, ligand exchange is a slow process because there is a large loss of ligand field stabilization energy when a ligand is removed from an octahedral d^6 complex (see Huheey et al., *Inorganic Chemistry: Principles of Structure and Reactivity*, 4th Ed. HarperCollins, N.Y., chapter 13). Generally, the
25 exchange is slow; for example, $[\text{Co(III)(acacen)(NH}_3)_2]\text{Cl}$ in water with excess

imidazole exchanges ammonia for imidazole with a half-life under an hour at 25 °C, with the rate of exchange increasing with temperature. However, reduction to cobalt(II) puts an electron into the antibonding d_{z^2} orbital, labilizing the axial ligands. Typical one-electron reduction potentials with irreversible loss of an axial ligand are around -360 mV vs NHE (Darbieu et al.,
5 Transition Met. Chem., 7:149 (1982)). This property may be exploited as a "redox switch" to control the activity of the cobalt compound. For example, certain regions within tumors are often oxygen-starved due to high metabolic demands and inadequate blood supply; therefore, reductive reactions might be
10 more favorable in such an environment than in a healthy cell (see A. C. Sartorelli, *Cancer Research*, (1988), 48, 775; Brown et al., J. Nat. Cancer Inst. 83:178 (1991)).

Raising the reduction potential of a cobalt acacen compound with substituents such as halides may place it high enough for reduction to occur readily in
15 tumor cells, but not in healthy cells. Ware and coworkers use a similar approach to attempt selective release of cobalt-bound cytotoxins in cancer cells (see Ware et al., *supra*).

Testing the efficacy of the cobalt compounds as inhibitors is routine, as will be appreciated in the art. When the target protein is an enzyme, testing is similar
20 to testing any enzyme inhibitor, as is known in the art. Generally, the enzyme is assayed in the presence and absence of the putative inhibitor, and kinetic parameters are calculated as is known in the art.

The amount of cobalt compound inhibitor needed to inhibit a given enzyme will vary depending on the number of other reactive axial ligands on the
25 surface of the enzyme, as is outlined above for protein labeling. For example, an enzyme with an active site histidine and two other "surface" histidines will generally require at least a 3:1 ratio of cobalt compound inhibitor:enzyme. The

total amount bound to the enzyme may be determined spectrophotometrically, as outlined above.

In a preferred embodiment, the Co(II) compound inhibitors are generated *in situ* by reducing the corresponding Co(III) compound. By "corresponding Co(III) compound" herein is meant a Co(III) compound which has the identical R groups as the Co(II) compound. Generally, the Co(III) compounds are synthesized with axial ligands, such as, but not limited to, amines, 2-methylimidazole, and water.

In this embodiment, the Co(III) compound is synthesized, and then added to the enzyme under conditions which can result in the reduction of the Co(III) to Co(II). This may be done in several ways. For example, the *in situ* environment of the enzyme, whether it be *in vitro* or *in vivo*, may be a reducing environment for the Co(III) compound, such that the Co(III) is reduced to Co(II). Alternatively, the Co(III) compound may contain an electron acceptor group as one of the R groups, such that in a given *in situ* environment, the electron acceptor group will pick up an electron and donate the electron to the Co(III), thus reducing it to the Co(II) form. Suitable electron acceptor groups include, but are not limited to, cations such as methyl viologen (N-N-dimethyl 4,4' bipyridine), or ethyl or propyl viologen, as is understood in the art.

Additionally, the reduction potential of the compound may be tailored such that introducing the compound into a particular environment causes reduction; for example, by glutathione in physiological systems. The resulting Co(II) compound then reacts with the reactive axial ligands of the enzyme to inhibit the enzyme as outlined above. Thus, the Co(II) compound is generated *in situ*; that is, a Co(III) compound is added to an enzyme, is reduced to the Co(II) form, which in turn inhibits the enzyme. In this embodiment, the Co(III) compounds may be inert with respect to a selected enzymatic target in a given

oxidation state, yet inactivate the enzyme target in a second oxidation state. This mechanism allows the *in situ* addition of a cobalt compound, whether *in vitro* or *in vivo*, in an inactive form, with activation to the Co(II) compound form in a particular reducing environment.

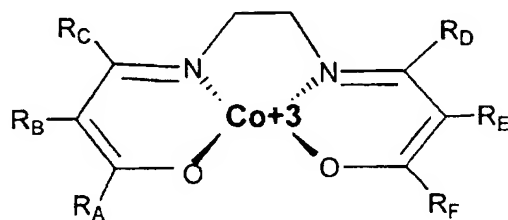
5 The compounds of the present invention may be formulated into pharmaceutical compositions, and administered in therapeutically effective dosages. By "therapeutically effective dose" herein is meant a dose that produces the effects for which it is administered. The exact dose will depend on the disorder to be treated and the protein to be inhibited, and will be
10 ascertainable by one skilled in the art using known techniques. In a preferred embodiment, the pharmaceutical compositions of the invention are in a water soluble form, and contain a pharmaceutically acceptable carrier in addition to the cobalt compound. The pharmaceutical compositions may be administered in a variety of ways, including, but not limited to, orally, subcutaneously,
15 intravenously, intraperitoneally, or topically.

Also provided are methods for inhibiting a selected protein or enzyme with the cobalt compounds of the invention. In this embodiment, the target protein is contacted or exposed to a cobalt compound. In a preferred embodiment, the cobalt compound has the structure depicted in Formula 1. The cobalt
20 compound can be targetted to a particular protein by the addition of a targeting moiety, such as a polypeptide or a nucleic acid.

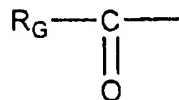
Also provided are methods for inhibiting a zinc finger protein, comprising contacting a zinc finger protein with a cobalt compound. By "inhibiting a zinc finger protein" herein is meant that the biological activity of the zinc finger
25 protein is decreased or eliminated upon exposure to the cobalt compound. Generally, when the zinc finger protein is a nucleic acid binding protein, this

means that the zinc finger will no longer bind the nucleic acid to a significant degree. Various prior art Co(III) compounds are well known in the art, (see U.S. Patent Nos. 4,866,054, 4,866,053, 5,049,557, 5,106,841, 5,142,076, and 5,210,096). These compounds, depicted below in Formula 7, as well as the compounds embodied in Formula 1, have utility in the inactivation of zinc finger proteins. Accordingly, in this embodiment, when Co is Co(III), there is no requirement that at least one of R₁ to R₈ is a targeting moiety such as a polypeptide or a nucleic acid, although this is preferred. Likewise, when Co is Co(II), there is no requirement that at least one of the R groups is hydrophilic, although this is preferred.

Formula 7



In Formula 7, R_A and R_F are the same or different and each is an alkyl group, a phenyl group or a substituted derivative of a phenyl group. R_B and R_E are the same or different and each is hydrogen, an unbranched alkyl group, a halide or a group having the structure:



wherein R_G is hydrogen, an alkoxide group, an alkyl group, or OH. R_C and R_D are the same or different and each is hydrogen or an alkyl group.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated

for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by reference.

5

EXAMPLES

Example 1

Synthesis of Cobalt Compounds

A sample of $[\text{Co}^{\text{III}}(\text{acacen})(\text{NH}_3)_2]\text{Cl}$ was obtained as a gift from Zvi Dori. Acetylacetone, benzoylacetone, ethylenediamine, and triethylamine (TEA) were obtained from Aldrich (Milwaukee, WI). Tris(hydroxymethyl)aminomethane (Tris, Trizma Base), polyethylene glycol (PEG 8000) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) were obtained from Sigma (St. Louis, MO). N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) was from J. T. Baker (Phillipsburg, NJ). Cobaltous acetate tetrahydrate was obtained from EM Science (Gibbstown, NJ). Human α -thrombin and the assay agent Spectrozyme TH (H-D-hexahydrotyrosyl-L-alanyl-L-arginine-p-nitroanilide diacetate) were purchased from American Diagnostica (Greenwich, CT). Antithrombotic peptides were manufactured as amides by the Beckman Institute Biopolymer Synthesis group at Caltech using solid phase methods. Weak cation exchange resin Sephadex G-25 was from Pharmacia (Uppsala, Sweden). Enzyme reactions were followed spectrophotometrically using a photodiode array spectrophotometer. Ultrafiltration materials were from Amicon (Beverly MA). HPLC used Vydac reverse phase columns. ^1H NMR were obtained on a 300 MHz FT-NMR spectrometer. Solvents used include EM Omnisolve MeOH, Omnisolve CH_2Cl_2 , passed over basic alumina to

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remove residual acid, Fluka (Buchs, Switzerland) puriss. MeOH and dioxane, and Quantum Chemical (Tuscola, IL) absolute EtOH. Distilled water was prepared by a Barnstead Nanopure system. All other solvents were reagent grade.

5 Synthesis of hydroxypropyl acacen

To 200 mL of deoxygenated CH_2Cl_2 was added 10 mL of acetyl acetone (acac, 0.0974 mol) and cannulated into a 250 mL addition funnel, which was attached to a 500 mL 3-neck roundbottom flask containing 100 mL of deoxygenated CH_2Cl_2 and 32.6 mL ethylenediamine (en, 0.488 mol). The solution containing
10 the acac was added dropwise to the en solution. The reaction mixture was extracted with two 50 mL portions of 0.2 NaPi, pH 5.5. The organic layer was separated and placed in a -20°C freezer overnight. The resulting solution was filtered through fluted filter paper and the solvent was removed *in vacuo*. The compound was further purified using flash silica gel chromatography using
15 95:5:0.5 (v:v:v) CH_2Cl_2 :MeOH:Et₃N as the eluant. The resulting monoacacen was characterized by NMR.

Monoacacen (0.5 g, 3.5×10^{-3} mol) was dissolved in 5 mL of ethanol and 7-hydroxy-2,4-heptanedione (0.51 g, 3.5×10^{-3} mol) was added. The dione was synthesized as described previously (Detty, M.R. J. Org. Chem., 44:2073-2077
20 (1979)). The reaction was allowed to proceed for 4 hours and the solvent was removed *in vacuo*. The sample was purified using flash silica gel chromatography using 93:7 (v:v) CH_2Cl_2 :MeOH as the eluant. The resulting hydroxypropyl acacen was characterized by NMR.

Synthesis of Co(II)hydroxypropyl acacen

25 Hydroxypropyl acacen (0.25 g, 9.4×10^{-4} mol) was dissolved in 2 mL of deoxygenated methanol in an inert atmosphere glove box. To this solution was

added $\text{Co(II)(CH}_3\text{COO-)}_2(\text{H}_2\text{O})_4$ (0.2338 g, 9.4×10^{-4} mol). The mixture was allowed to stir for an additional thirty minutes. The reaction vessel was sealed and the solvent was removed *in vacuo*. The compound was used without further purification.

5 Synthesis of $[\text{Co(III)hydroxypropylacacen}(\text{NH}_3)_2]\text{CH}_3\text{COO}$

Hydroxypropyl acacen was reacted with Co(acetate) as described earlier. However after the reaction vessel was sealed, anhydrous ammonia gas was bubbled through the reaction mixture and subsequently exposed to air. The solvent was removed *in vacuo*, and the product was purified using an alumina
10 column with neat methanol as the eluant. The sample was characterized by NMR.

Synthesis of Acacen

To 20 mL of ethanol was added 20 mL of acac (0.0973 mol). To this solution was added 6.5 mL of ethylenediamine (0.0973 mol) using an addition funnel.
15 The solution was placed in a refrigerator at 4°C overnight, and the crystals were triturated three times with anhydrous diethylether (MP = 110.1-111.1).

Synthesis of $[\text{Co(III)acacen}(\text{NH}_3)_2]\text{Cl}$

249.08 g of cobalt acetate, 6 H_2O , (1 mol) was dissolved in 1.750 L methanol and the solution was filtered through Whatman paper No. 1. Acacen (1 mol)
20 was suspended in 150 mL methanol. Nitrogen dried by passage through a silica gel dessicant column was bubbled over the reagents for 15 minutes. The cobalt acetate solution was added dropwise (1/2 hour) and the orange-brown solution was left to react at room temperature under nitrogen for 2 hours. The flask was opened to air and NH_3 gas was bubbled into the solution; the mixture
25 was concentrated on a rotary evaporator. An equivalent of sodium chloride dissolved in a minimum amount of water was added, poured into a wide vessel,

and left to crystallize slowly. The brown crystalline powder was filtered, washed with methanol and dried.

Further synthesis of assymetrical or "mixed" ligands

5 "Acacen" (compound 9 in Scheme II): 1 equivalent ethylenediamine in anhydrous EtOH was added to 2 equivalents acetylacetone in EtOH with stirring. After 30 minutes, the mixture was put in the freezer to precipitate a white crystalline solid. The product was collected by vacuum filtration over a coarse glass frit and rinsed with diethyl ether. It can be recrystallized from benzene to desired purity. Purified crystals melted at 111°C.

10 "Monoacacen" (compound 7 in Scheme II): The 1:1 condensation product of acac and en was prepared according to literature procedures (Cros et al., C.R.Acad. Sc., Ser. II 294:173 (1982)) substituting CH_2Cl_2 for chloroform. The resulting yellow oil often contained some acacen (about 10%), which could be removed by flash chromatography on silica using 97 CH_2Cl_2 / 3 MeOH/ 0.5 TEA either now or after the addition of another diketone.

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"Bzacacacen"(the Formula 1 compound with R1, R3 and R6 as methyl, R2 and R7 as hydrogen, and R8 as phenyl) : 1 equiv. benzoylacetone in CH_2Cl_2 was added to a solution of monoacacen in CH_2Cl_2 . Removal of solvent gave a white powder containing some acacen impurity. Purification was accomplished by

20 flash chromatography on silica using 97 CH_2Cl_2 / 3 MeOH/ 0.5 TEA.

"Aciden" (compound 11 in Scheme III): A solution of 1 equiv. 4,6-dioxoheptanoic acid in CH_2Cl_2 was added to 1 equiv. ethylenediamine in CH_2Cl_2 and the insoluble 1:1 condensation product immediately precipitated. The product was collected over a frit and dried in vacuo. The melting point was

25 140°C, with decomposition. A direct reaction of 4,6-dioxoheptanoic acid with

monoacacen did not work, despite repeated attempts. Evidently, the acid group was effecting decomposition, even under anhydrous conditions. Nor did using excess triethylamine to neutralize the diketoacid give satisfactory results.

5 "Acacaciden" (compound 13 in Scheme III): 1 equiv. aciden was powdered and slurried in Fluka puriss. MeOH. 1 equiv. triethylamine and 2-2.5 equivalents acac were added and the mixture was allowed to stir overnight to give a yellow solution. It was evaporated to dryness to obtain the crude product as an orange oil. Further purification by flash chromatography over silica using a 5% to 25% gradient of MeOH in CH₂Cl₂ with 0.5% TEA to guard against hydrolysis of
10 imine bonds. Evaporation of solvent followed by recrystallization from EtOH gave a beige solid. M' was 282, as expected.

[Co(III)(acacen)(NH₃)₂]Cl : Procedure obtained from Zvi Dori (The Technion, Haifa, Israel). 1 equiv. of acacen was degassed *in vacuo* and placed under argon. Dry, degassed methanol was transferred into the flask via cannula. 1
15 equiv. cobaltous acetate was treated in same manner and the resulting purple solution added via cannula to the clear solution of the ligand. An immediate color change from purple to orange was observed as the reaction was stirred under argon for two hours. Ammonia gas was bubbled into the solution and the flask opened to air. Reaction was stirred with ammonia for 4 hours, evaporating
20 solvent replenished as necessary. The reddish solution was filtered over a frit and concentrated on a hot plate. Addition of saturated aqueous NaCl precipitates the brown product. It can be recrystallized from ethanol to give a tan powder.

[Co(III)(acacaciden)(NH₃)₂]: The above metallation conditions were used, but
25 with the acacaciden ligand. Crude reaction mixture did not afford precipitate, but purification over cation exchange resin using aqueous ammonium acetate

followed by removal of the volatile buffer gave a light brown powder. M^+ was 373, as expected.

Peptide synthesis of GGGdFPRamide: The peptides were synthesized by the Beckman Institute Biopolymer synthesis group (Caltech). This was accomplished on p-methylbenzhydrylamine (MBHA) resin using N-*tert*-butyloxycarbonyl (Boc) amino acid derivatives for Merrifield solid-phase synthesis on an ABI Model 430A peptide synthesizer. The terminal Boc protecting group was removed with trifluoroacetic acid (TFA). Side chain protecting groups and the peptide-resin bond were cleaved under HF conditions (90% HF, 5% *p*-cresol, 5% *p*-thiocresol). After removal of HF under vacuum, the peptide/resin mixture was washed on a fritted funnel with ether. The peptide was then dissolved in 10% aqueous acetic acid and filtered through, leaving the resin behind. The crude peptide solution was subjected to gel filtration on anion exchange resin AG 1-X2 to remove the scavengers. The peptide can be further purified by reversed-phase HPLC on a Vydac C8 column using a 30-min. linear gradient of 6-26% acetonitrile/water/0.1% TFA with a 2.0 mL/min. flow rate.

Coupled product $[\text{Co(III)}(\text{acacen-GGFPR})(\text{NH}_3)_2]$; shown in Figure 2]: One potential difficulty in coupling the cobalt complex to this peptide is that the peptide's arginine side chain is more reactive than its N-terminus if the arginine is not protected or protonated (Bodzansky, *Peptide Chemistry: A Practical Textbook*, Springer-Verlag, Berlin, 1988). Since arginine has a pK_a around 12.0, it is easily protonated, but this renders the hydrophilic peptide insoluble in the organic solvents, such as dioxane, used for most coupling reactions. For this reason, we used the water-soluble coupling reagent 1-(3-dimethylamino-propyl) -3-ethylcarbodiimide (EDC). At least a 10-fold excess of EDC is needed to compensate for its hydrolysis over the course of the

reaction. The large quantity of urea byproduct generated can be reduced by passing the solution through an Amicon YC05 filter or by extracting the crude oil with an organic solvent.

5 In addition, HPLC purification of a basic, hydrophilic peptide usually calls for a small amount of an organic acid in the eluting solvent to aid retention. Since such an acid would attack the imine bonds of the free ligand, purification was attempted without it, but neither reverse phase C8 and C18 nor normal phase cyano columns were effective in resolving the mixture. Later, some progress was made using basic ammonium acetate buffer and acetonitrile on reversed
10 phase, but there still was some decomposition of the product due to hydrolysis of the imine bonds. In order to prevent this, the imines were protected by inserting the metal into the ligand before attaching the peptide.

Synthesis was as follows. $[\text{CoIII}(\text{acacaciden})(\text{NH}_3)_2]$ was dissolved in 0.1 M HEPES buffer, pH 8 at 5°C. 1 equiv. peptide dissolved in the same buffer was
15 added. 5 equiv. EDC were added directly. 4 hours later, another 5 equiv. were added. The reaction was stirred overnight at 5°C, then lyophilized to give a reddish brown product. The crude material was purified over cation exchange resin (Pharmacia G-25), eluting with ammonium acetate. Two products were collected, both of which contained the cobalt, based on reddish color, and the
20 peptide, based on the presence of the phenylalanine signals in the ^1H NMR spectra (multiplets at 7.28 and 7.20 ppm). Mass spectrometry of both materials suggested that the first of the two to elute had lost an axial ligand, possibly replaced by coordination of the metal to arginine. This would likely deprotonate the arginine, lowering the overall charge to +1, causing it to elute
25 earlier. Mass spectrometry also suggests that the second band contains the desired product, as the first had $M^+ = 932$, the second had $M^+ = 1066$. The calculated mass of the desired product as a diacetate salt is 1062.

Example 2

Inhibition of Carbonic Anhydrase

Inhibition of Co(III) compound

Bovine carbonic anhydrase (20 mg, 6.7×10^{-7} mol, Calbiochem) was dissolved
 5 in 0.5 mL Tris buffer (pH = 8, 0.05 M). To this solution was added 30 mg of
 Co(III)hydroxypropyl acacen (30 mg, 7.6×10^{-5} mol) dissolved in 0.5 mL of
 H₂O. This solution was incubated for 48 hours. The excess cobalt complex
 was separated from the protein using a PD-10 gel filtration column equilibrated
 with Tris buffer (pH 8, 0.05 M). This enzyme, which was incubated with the
 10 Co(III) complex, retained 100% of its activity.

Inhibition with Co(II) compound

Two samples of bovine carbonic anhydrase (30 mg, 1×10^{-6} mol) were
 dissolved in 3 mL of degassed Tris buffer (pH 8, 0.05 M). To one of the
 samples was added Co(II)hydroxypropyl acacen (30 mg, 9×10^{-5} mol). The
 15 other sample of bovine carbonic anhydrase served as the control. The solutions
 were incubated under inert atmosphere (glove box) and a 1 mL aliquot was
 removed from each sample after 48 and 96 hours of incubation.
 The protein was then exposed to air and the excess Co(II)hydroxypropyl acacen
 separated from the protein using a PD-10 gel filtration column (Pharmacia)
 20 equilibrated with Tris buffer (pH 8, 0.05 M). The enzymatic activity of the
 protein was assayed using p-nitrophenylacetate as the substrate (Pocker et al.,
 Biochem. 6:668-678 (1967)). The results are shown below:

Inhibition of Carbonic Anhydrase (CA) with
 Co(II)hydroxypropyl acacen

25	Time of incubation	% inhibition of CA
	48 hours	33.8%

96 hours

43.2%

Example 3

Inhibition of Thermolysin

Thermolysin (2,500,000 units, Calbiochem) was dissolved in 20 mL of Tris
 5 buffer (pH 7.2, 0.1 M, 2.5 M NaBr, 0.01 M CaCl_2) and stored at 4°C; enzyme
 concentration was determined by using $E_{1\%}^{280} = 17.65$ and a molecular weight
 of 34,600. This solution was further purified using gel filtration
 chromatography on an FPLC using a Superdex 75 column (Pharmacia)
 equilibrated with 0.1 M Tris, 0.1 M NaBr, 0.01 CaCl_2 , pH 7.2. This stock
 10 solution was stored at 4°C. N-[3-(2-furyl)acryloyl]glycyl L-leucinamide
 (FAGLA) was obtained from Sigma as the thermolysin substrate. A stock
 solution of FAGLA (4.0 mM) was prepared by dissolving the substrate in
 dimethylformamide (DMF) and diluting it with buffer to a final concentration of
 0.1 M Tris, 0.1 NaBr and 10 mM CaCl_2 , pH 7.0 (final concentration of DMF
 15 was 2.5%; see Feder et al., Biochem. 9:2784-2791 (1970)). For all assays, the
 concentration of enzyme and substrate was 50 nM and 2.0 mM respectively.
 The peptidase activity of thermolysin was determined by following the
 decrease in absorption at 346 due to the enzymatic hydrolysis of FAGLA.
 Initial velocities were determined for $\leq 10\%$ of the reaction.

20 Thermolysin (2×10^{-5} mol) was incubated with $[\text{Co(III)acacen}(\text{NH}_3)_2]\text{Cl}$ (5
 mM) in HEPES buffer (pH 7.0, 0.01 M, 0.005 M CaCl_2). The concentration of
 thermolysin was 5×10^{-8} M, while the concentration of the cobalt inhibitor was
 1.25×10^{-5} M. The results of this study are shown below and in Figure 3:

Inhibition of Thermolysin

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Time of incubation

% inhibition

45 minutes	46.2%
190 minutes	63.9%
322 minutes	77.7%

5 Stock solution of thermolysin was mixed with the cobalt compound dissolved in 0.1 M Tris, 0.1 M NaBr, 0.01 M CaCl_2 , pH 7.2 (run buffer) to yield a final enzyme concentration of 10 mM and a cobalt concentration of 2.5 mM. These solutions were incubated at 25°C or 37°C for several hours along with a control lacking cobalt compound. Periodically 5 ml aliquots of these solutions were assayed for residual enzyme activity by their addition to a cuvette containing 495
10 mL of run buffer and 500 μL of FAGLA stock solution, and following the absorption decrease at 346 nm as described above. All enzyme assays were performed at 25°C. The results are shown in Figure 3.

Ligand exchange experiments are shown in Figure 3B. This experiment models the binding of the cobalt compounds to histidine residues on
15 thermolysin by monitoring the binding of imidazole to the bisamine product ($\text{Co(III)acacen(NH}_3)_2$). 1.35 mM of $\text{Co(III)acacen(NH}_3)_2$ was incubated with 0.1 M imidazole in run buffer. The rise in absorbance at 420 nm due to the exchange of NH_3 with imidazole was monitored with time at 25°C and 37°C. The similarity in the temperature dependence of enzyme inhibition and ligand
20 exchange in the model cobalt complex suggests that ligand exchange with a histidine residue is the rate limiting step of enzyme inhibition.

The binding of the cobalt compound to the active site histidine was confirmed as follows. Thermolysin (10 μM in running buffer (0.1 M Tris, 0.1 M NaBr, 0.01 M CaCl_2 , pH 7.2) was incubated at 25°C with $\text{Co(III)(acacen)(NH}_3)_2\text{Cl}$ (5
25 mM) in the presence and absence of the inhibitor phosphoramidon (N- α -L-rhamnopyransyloxyphospho-L-leucyl-L-tryptophan, 50 μM), which has a

reported K_i of 32 nM at pH 7.5 (Kitagishi et al., J. Biochem. 95:529-534 (1984). Phosphoramidon binds to thermolysin at the active site, and this enzyme-inhibitor complex has been crystallographically characterized (Weaver et al., J. Mol. Bio. 114:119-132 (1977)). After incubation with the cobalt compound overnight, the inhibitor was separated from the enzyme using gel filtration chromatography on an FPLC using a Superdex 75 column (Pharmacia) equilibrated with 0.1 M Tris, 5 mM CaCl_2 , pH 9. The resulting solution was transferred into the Tris running buffer using a PD-10 column (Pharmacia), and was characterized. There was no detectable loss of enzyme activity due to irreversible inactivation by the cobalt complex after removal of the inhibitor. Spectrophotometric characterization of this active enzyme revealed the binding of two cobalt complexes to the enzyme. Characterization of thermolysin, completely inactivated by the cobalt complex, showed the binding of three equivalents of the cobalt compound for each enzyme molecule. Since protection of the active site prevents inhibitor of thermolysin, and it prevents the binding of one equivalent of cobalt compound to the enzyme, the inhibition of the enzyme is a consequence of the binding of one cobalt compound at the enzyme active site.

Example 4

Inhibition of Thrombin

Thrombin was chosen as the first target enzyme. Several crystal structures of thrombin are available from the Protein Data Bank (e.g. file 1PPB; see Bode et al., EMBO J. 8:3467 (1989)). Thrombin is a 34 kD serine protease with a well defined mechanism of action involving a histidine residue. It is vital to the coagulation cascade, but an unwanted clot is a severe, life-threatening condition. Thrombin was chosen for this investigation because its structure and

mechanism are well understood, there is a simple activity assay, and antithrombotic drugs are useful in the treatment of strokes.

Thrombin inhibition assay: As with any purified blood product, proper care was taken to avoid the transmission of blood-borne pathogens. Thrombin was taken as received (about 1 mL at 30 μ M in 0.75 M sodium chloride storage solution) and divided into 100 μ L aliquots. Each aliquot was diluted to 10 mL using clean, filtered (2 μ m) aqueous 0.75 M NaCl and divided into 1 mL samples. Each sample was stored frozen at -80°C until ready for use. The protein should not be stored at -20 °C as this is too close to the eutectic point for the thrombin-salt mixture and freeze-thaw cycling may damage the protein.

An assay buffer containing 10 mM Tris, 10 mM HEPES, 0.1% polyethylene glycol (PEG 8000) and 500 mM sodium chloride was prepared to pH 8. Following manufacturer's instructions, 5 μ mole Spectrozyme TH was dissolved in 1.000 mL filtered nanopure water. A series of [Co^{III}(acacen)(NH₃)₂]Cl solutions was prepared by dissolving 16.6 mg of the compound in 0.75M NaCl and then diluting aliquots to a range of cobalt concentrations from 4.7 mM to 4.7 nM. Both of the purified materials were assayed.

Thrombin was preincubated with inhibitor in the kinetics buffer (total volume of thrombin, buffer, and inhibitor of 992mL) for the times specified in Figure 1. After incubation, the substrate, spectrozyme TH was added (8mL of 5mM spectrozyme), and the thrombin-catalyzed hydrolysis rates were monitored at 406 nm. The final concentration of thrombin in the experiments was 3 nM, and the concentration of substrate was 40 mM. The inhibitor concentrations are outlined in Figure 1. The rates of hydrolysis were determined from the linear portion of the saturation-kinetics plots. The percent activity is determined by

dividing the rate of spectrozyme hydrolysis with inhibitor by the rate of hydrolysis without inhibitor and multiplying by 100.

A vial of thrombin prepared as above was thawed in warm water. 100 μL aliquots were added to 100 μL samples of the cobalt acacen solutions. One 100 μL aliquot of thrombin solution was diluted with 100 μL 0.75M NaCl to be used as a control. Samples were incubated as needed before assay. 980 μL of the assay buffer was placed in a 1.0 mL, 1 cm quartz cuvette and allowed to equilibrate to 25 °C (Hewett-Packard Peltier constant temperature cell holder). 10 μL of a sample was mixed thoroughly into the cell's contents. The spectrophotometer was set for a 30 second delay during which 10 μL Spectrozyme TH solution was added and mixed into the cell's contents by inverting the capped cell a few times before replacing it in the spectrophotometer. Scans from 250 to 500 nm were taken every 30 seconds for 10 minutes, although a single-wavelength scan at 406 nm would suffice. After the runs, the control was allowed to hydrolyze to completion before determining the end point.

The absorbance values at 406 nm were extracted and used to find the pseudo-first order rate constant according to the formula:

$$-\ln[(A_{\infty}-A_t)/(A_{\infty}-A_0)] = kt$$

where A_{∞} is the absorbance at completion, A_0 is the initial absorbance (approximately the first data point) and A_t is the absorbance at each time, t . The slope of the linear fit (typically $R^2 > 0.99$) yields k . Comparison to an uninhibited control sample gave an indication of the relative activity for each cobalt-containing sample. Controls were repeated periodically as a check for protein degradation.

First the inhibition of thrombin by unmodified $[\text{Co}^{\text{III}}(\text{acacen})(\text{NH}_3)_2]\text{Cl}$ was investigated. A solution containing $0.1 \mu\text{M}$ thrombin and 2.5 mM $[\text{Co}^{\text{III}}(\text{acacen})(\text{NH}_3)_2]\text{Cl}$ was incubated at room temperature for 24 hours. As a control, thrombin from the same source was incubated without inhibitor for the same length of time. A portion of each incubated solution was assayed at 25°C with an excess of a commercial substrate, Spectrozyme TH, whose proteolysis releases a chromophore, p-nitroaniline. The pseudo-first order production of p-nitroaniline was monitored spectrophotometrically and the rate constant extracted for each run. The activity of the control sample was normal, but the cobalt-containing sample was completely inactive (Figure XX). The cobalt-free ligand had no effect.

The same experiment was set up under an inert atmosphere, using a solution of water soluble $\text{Co}(\text{II})$ hydroxypropylacacen, at similar concentrations as before. After incubation, both sample and control were exposed to air to oxidize the cobalt. Again loss of activity was found for the cobalt-containing sample. Surprisingly, the $\text{Co}(\text{III})$ compound was a more effective inhibitor (0% activity) than the $\text{Co}(\text{II})$ compound (42% activity relative to its control). Perhaps the overall positive charge on the $\text{Co}(\text{III})$ compound assists in attracting the inhibitor to the active site as is seen in a different system (Bagger, J. Inorg. Biochem. 52:165 (1993)).

The $\{\text{Co}(\text{III})\text{acacen}(\text{NH}_3)_2\}\text{Cl}$ experiments were repeated for a range of cobalt concentrations from 2.4 mM to 2.4 nM after at 12 hour incubation at 25°C (Figure XX). Cobalt concentrations as low as $24 \mu\text{M}$ were found to inhibit the protein. Incubation at a lower temperature, 5°C , slows the onset of thrombin inhibition, just as it slows the ligand exchange of $\text{Co}(\text{III})$. Partial inhibition was observed over a range of cobalt concentrations after 3 hours at 5°C . Activity was determined at 25°C as before and was found to decrease with

higher cobalt concentrations and longer incubation times. The activity of the control sample lacking the cobalt compound was stable over time.

5 A crude attempt to determine the number of cobalt complexes bound to the enzyme was made by first passing the inhibited protein down a size exclusion column to remove most of the unbound cobalt complex and then determining the absorbances at two wavelengths for which the extinction coefficients of both pure thrombin and cobalt compound were known; this information gives the concentrations of each based on Beer's law. The initial estimate for two separate samples is 5 - 8 cobalts per enzyme. There are only five histidines in
10 thrombin, but binding to other residues and electrostatic binding cannot be discounted.

Inhibition of Thrombin by Peptides Containing dPheProArg

The targeting approach requires attaching a recognition element to the cobalt complex for binding specifically to the active site of thrombin. The tripeptide
15 sequence dPhe-Pro-Arg is a known inhibitor of thrombin, the arginine binding tightly to the P1 aspartate. The peptides GGdFPR, GGGdFPR, GGFPR and GGGFPR were obtained as amides and assayed against thrombin. As expected from the crystal structures and inhibition data for similar peptides, (Bajusz et al., Int. J. Peptide Protein Res. 12:217 (1978)), the dPhe-containing peptides
20 were found to be better inhibitors since they can access a hydrophobic binding pocket more efficiently than the natural isomers. The K_i for (Gly)₃dPheProArg is about 209 μ M.

Both purified Co(III)-peptide compounds (the two peaks from cation exchange resin) were assayed against thrombin without additional purification, the
25 concentrations of each determined by assuming that the extinction coefficients were similar to that of $[\text{Co}^{\text{III}}(\text{acacen})(\text{NH}_3)_2]\text{Cl}$ (7,700 $\text{M}^{-1}\text{cm}^{-1}$). Preliminary

results indicate that both inhibit at concentrations less than 1 μ M, an order of magnitude lower than either $[\text{Co}^{\text{III}}(\text{acacen})(\text{NH}_3)_2]\text{Cl}$ or the peptide alone.

Example 5

Inhibition of a Zinc Finger Transcription Factor

5 In order to demonstrate that cobalt compounds can disrupt the binding of a zinc finger to its consensus sequence, two model systems were used. Human Sp1 transcription factor, which contains three CCHH zinc fingers and a synthetic peptide representing the first zinc finger region of retroviral nucleocapsid protein.

10 Inhibition of Sp1 Binding

Samples containing 20 μ l of binding buffer (25 mM Tris pH 8.0, 100 mM KCl, 2 mM DTT, 100 μ M ZnCl_2 , 10% glycerol) and 25 ng of Sp1 (Promega) were incubated with 40 fmol of ^{32}P labeled oligonucleotide in the presence or absence of cobalt chelate complex ($\text{Co}(\text{III})\text{acacen}$ with either NH_3 or imidazole as the axial ligands) at various concentrations (.001 to 0.05 mM) and evaluated by gel shift and filter binding assays.

20 Gel shift: Samples were run on a 4% polyacrylamide gel (80:1) at room temperature, 100V, in 0.5 X TBE. Gels had been prerun for 30 minutes prior to loading. These experiments demonstrated that the presence of cobalt complex inhibited the binding of Sp1 to consensus oligonucleotide.

Filter Binding Assay: The above samples were applied to nitrocellulose (0.45 μ m filters, Schleicher and Schuell) and washed twice with washing buffer (100 mM HEPES, pH 7.5, 1 mM EDTA). Membranes were incubated for 15 minutes at room temperature with Filtron-X (National Diagnostics) and bound

counts were detected by liquid scintillation (Beckman Instruments). Increasing amounts of cobalt compound resulted in decreased counts bound to the filter, indicating a loss of binding between Sp1 and oligonucleotide.

Disruption of the Structure of a Synthetic Retroviral Zinc Finger

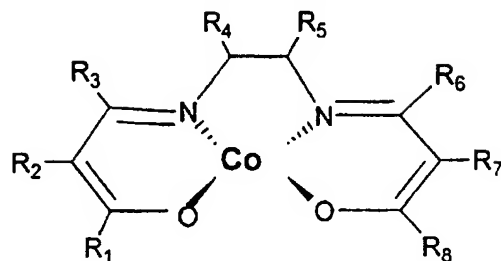
- 5 An 18 amino acid peptide corresponding to the first zinc finger region of the HIV nucleocapsid protein was synthesized and examined in a series of structural studies.

- 10 Confirmation of Zinc Finger Structure: 0.1 mg/ml solutions of peptide in 25 mM phosphate buffer pH 7.0 were examined by circular dichroism spectroscopy. In the absence of zinc, peptides displayed spectra characteristic of random coil structure. In the presence of zinc, the spectra changed dramatically to one indicative of type II turn content and zinc finger structure.

- 15 Disruption of Zinc Finger Structure: 4 mg peptide in 350 μ l D₂O was subjected to protein NMR spectroscopy. Spectra in the absence of zinc displayed multiple peaks in the aromatic region, including peaks representing protons from metal-free histidine. In the presence of zinc those peaks disappeared and were replaced by peaks representing protons from metal-bound histidine. The presence of cobalt compound resulted in a dramatically altered spectra indicating disturbance of the structure of the peptide.

CLAIMS

1. A compound having the formula comprising:



wherein

Co is either Co(II) or Co(III);

5 R₁ is hydrogen, alkyl, hydrophobic organic acid, alkyl amine, amine, alkyl alcohol, alcohol, or targeting moiety;

R₂ is hydrogen, alkyl, hydrophobic organic acid, alkyl amine, amine, alkyl alcohol, alcohol, or targeting moiety;

10 R₃ is hydrogen, alkyl, hydrophobic organic acid, alkyl amine, amine, alkyl alcohol, alcohol, or targeting moiety;

R₄ is hydrogen, alkyl, hydrophobic organic acid, alkyl amine, amine, alkyl alcohol, alcohol, or targeting moiety;

R₅ is hydrogen, alkyl, hydrophobic organic acid, alkyl amine, amine, alkyl alcohol, alcohol, or targeting moiety;

15 R₆ is hydrogen, alkyl, hydrophobic organic acid, alkyl amine, amine, alkyl alcohol, alcohol, or targeting moiety;

R₇ is hydrogen, alkyl, hydrophobic organic acid, alkyl amine, amine, alkyl alcohol, alcohol, or targeting moiety; and

20 R₈ is hydrogen, alkyl, hydrophobic organic acid, alkyl amine, amine, alkyl alcohol, alcohol, or targeting moiety;

wherein when Co is Co(III), at least one of R₁, R₂, R₃, R₄, R₅, R₆, R₇ and R₈ is a targeting moiety;

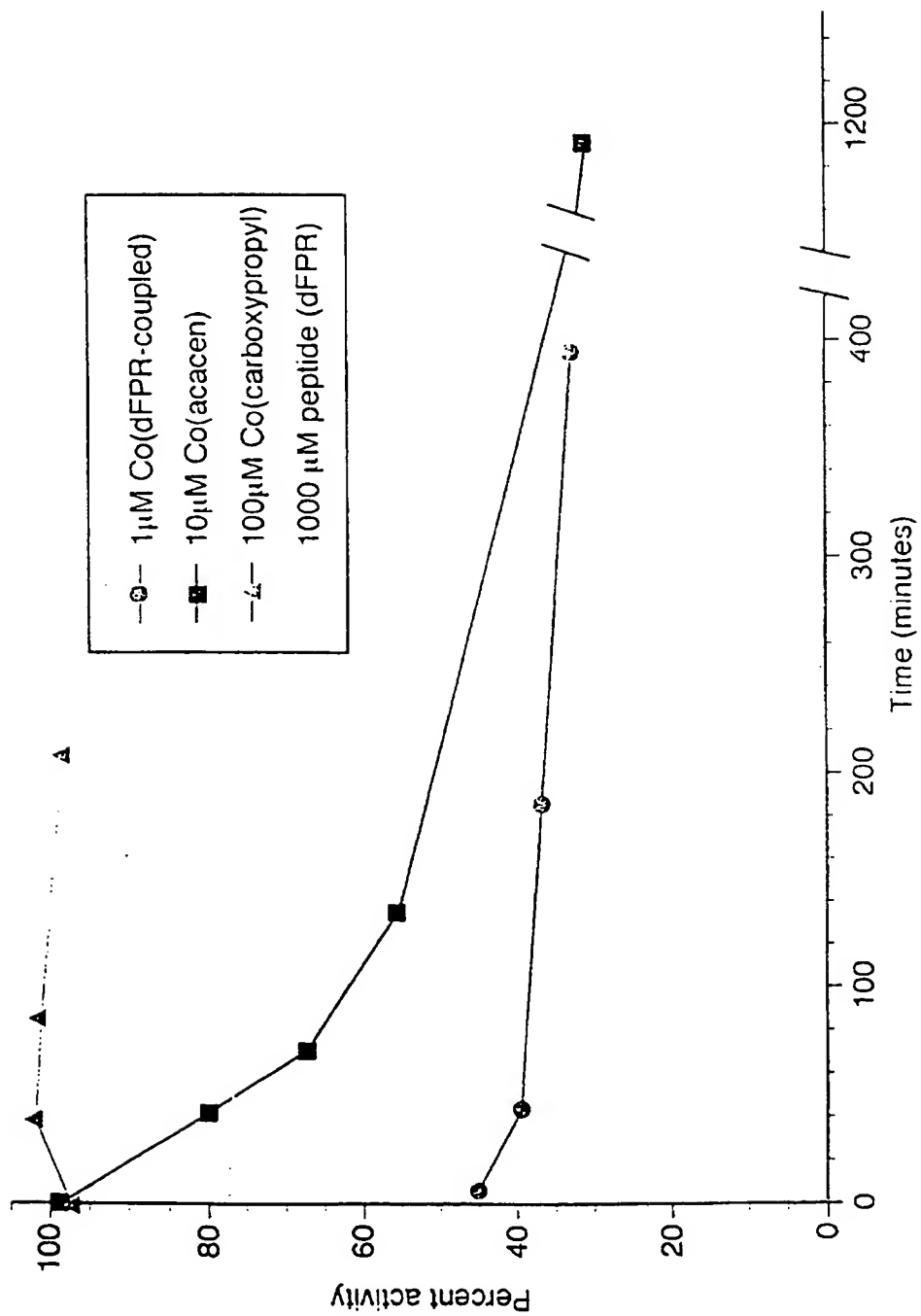
wherein when Co is Co(II), at least one of R₁, R₂, R₃, R₄, R₅, R₆, R₇ and R₈ is hydrophilic such that the compound is soluble in aqueous solution.

2. A compound according to claim 1 wherein Co is Co(II).
3. A compound according to claim 2 wherein at least one of R₁, R₂, R₃, R₄, R₅,
5 R₆, R₇ and R₈ is a targeting moiety.
4. A compound according to claim 3 wherein at least one of R₁, R₂, R₃, R₄, R₅,
R₆, R₇ and R₈ is polypeptide or a nucleic acid.
5. A compound according to claim 1 wherein Co is Co(III).
6. A compound according to claim 1 wherein R₁, R₂, R₃ and R₄ are each
10 hydrogen, alkyl or aryl.
7. A compound according to claim 1 further comprises a first axial ligand.
8. A protein-cobalt compound complex comprising a protein and a compound attached thereto wherein said cobalt compound has the structure shown in claim 1.
9. A complex according to claim 8 wherein said protein is an enzyme.
15
10. A method of inhibiting a selected protein comprising contacting said selected protein with the compound of claim 1.
11. A method according to claim 10 wherein said protein is an enzyme.

13. A composition according to claim 1 wherein R_1 is N-hydroxypropyl, R_2 is hydrogen, R_3 is methyl, R_4 is methyl, R_5 is hydrogen, R_6 is methyl, and R_{10} and R_{11} are hydrogen.
- 5 14. A composition comprising a water soluble tetradentate Schiff's base complex of Co^{+2} .
15. A composition according to Claim 1 wherein said compound further comprises a first axial ligand.

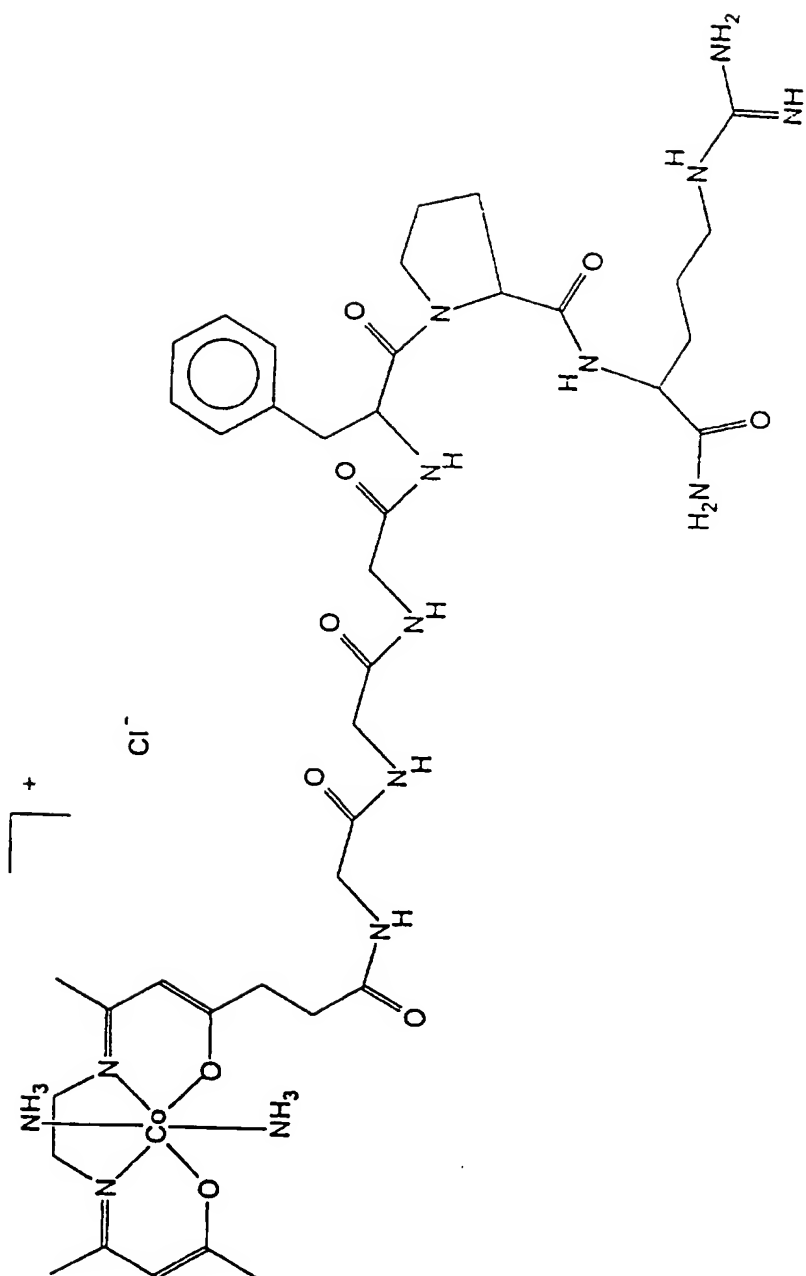
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Enzyme Targeted CIP: Figure 1



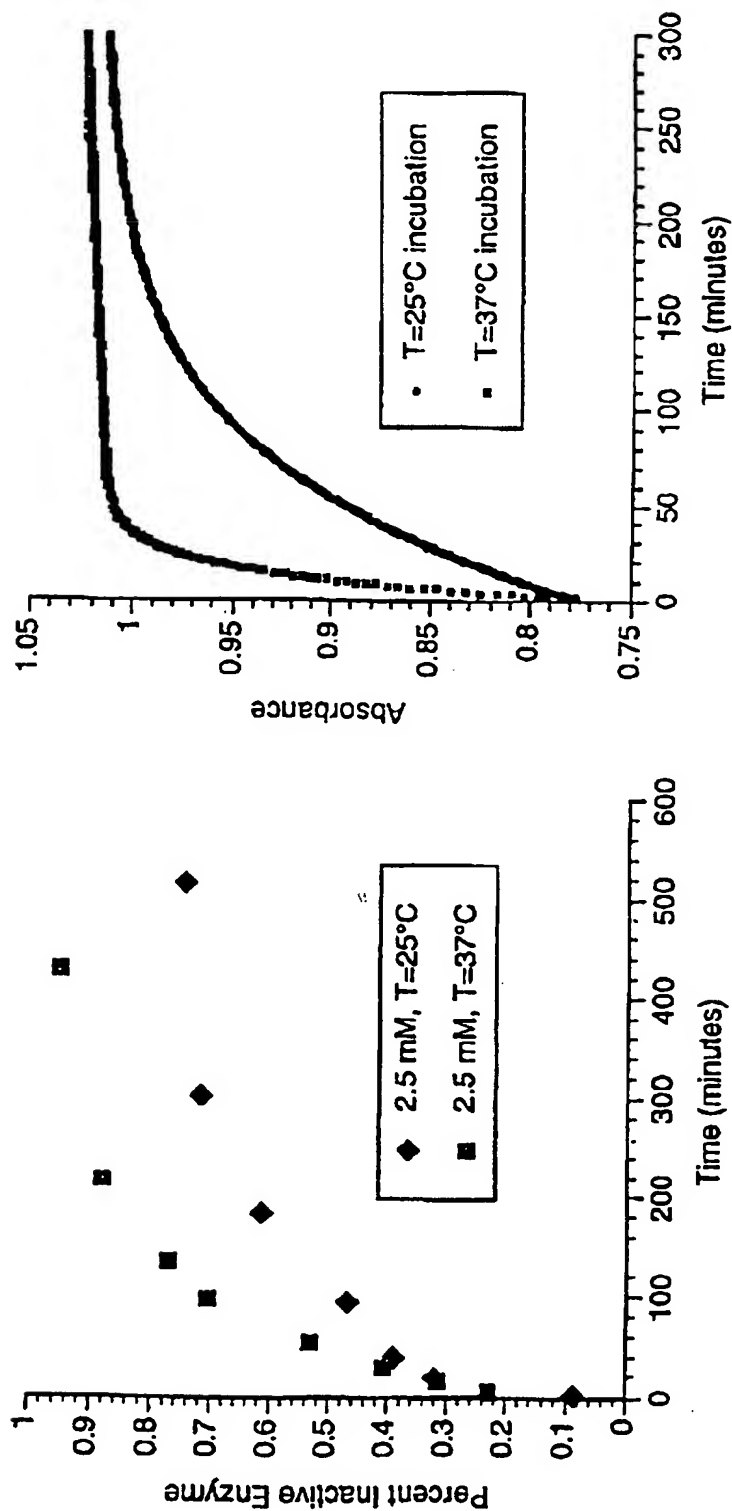
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Figure 2



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Temperature Dependence of Enzyme Inhibition Rate Correlates with Ligand Exchange Rate



Inhibition of Thermolysin with Co(acacen)(NH₃)₂ Exchange of NH₃ with Imidazole
 1.35 mM Co(acacen)(NH₃)₂ + 0.1 M Imidazole

A

B

Figure 3

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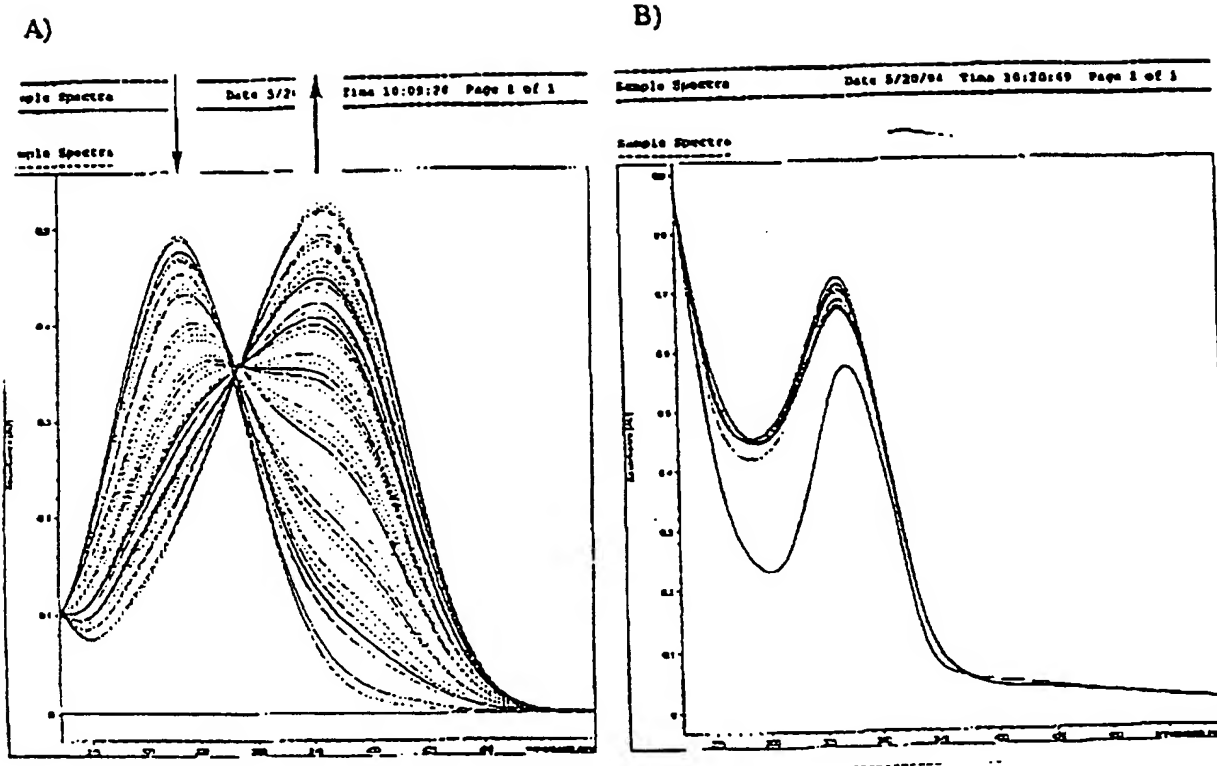


Figure 4

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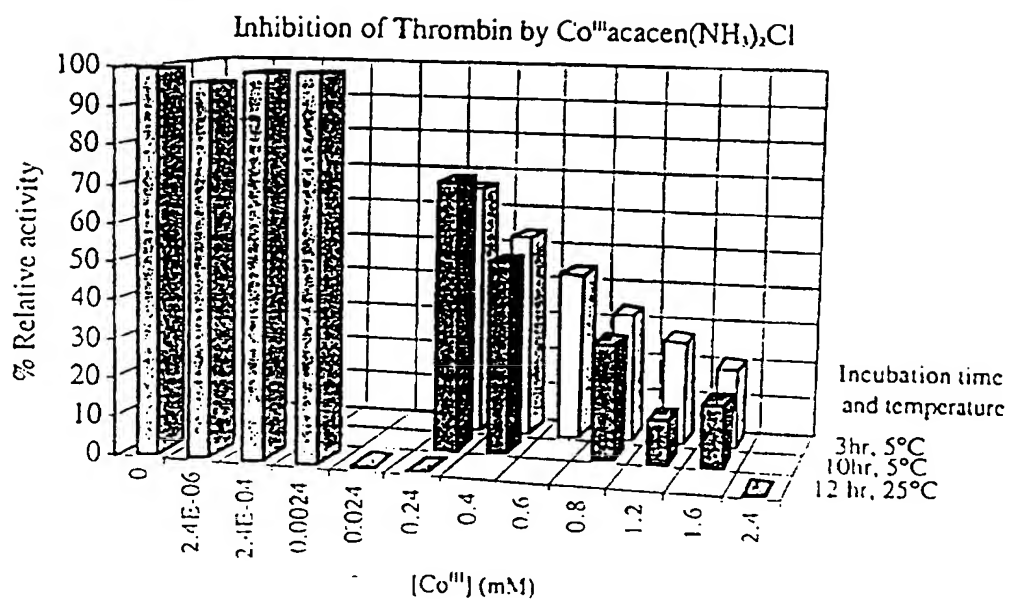


Figure 5

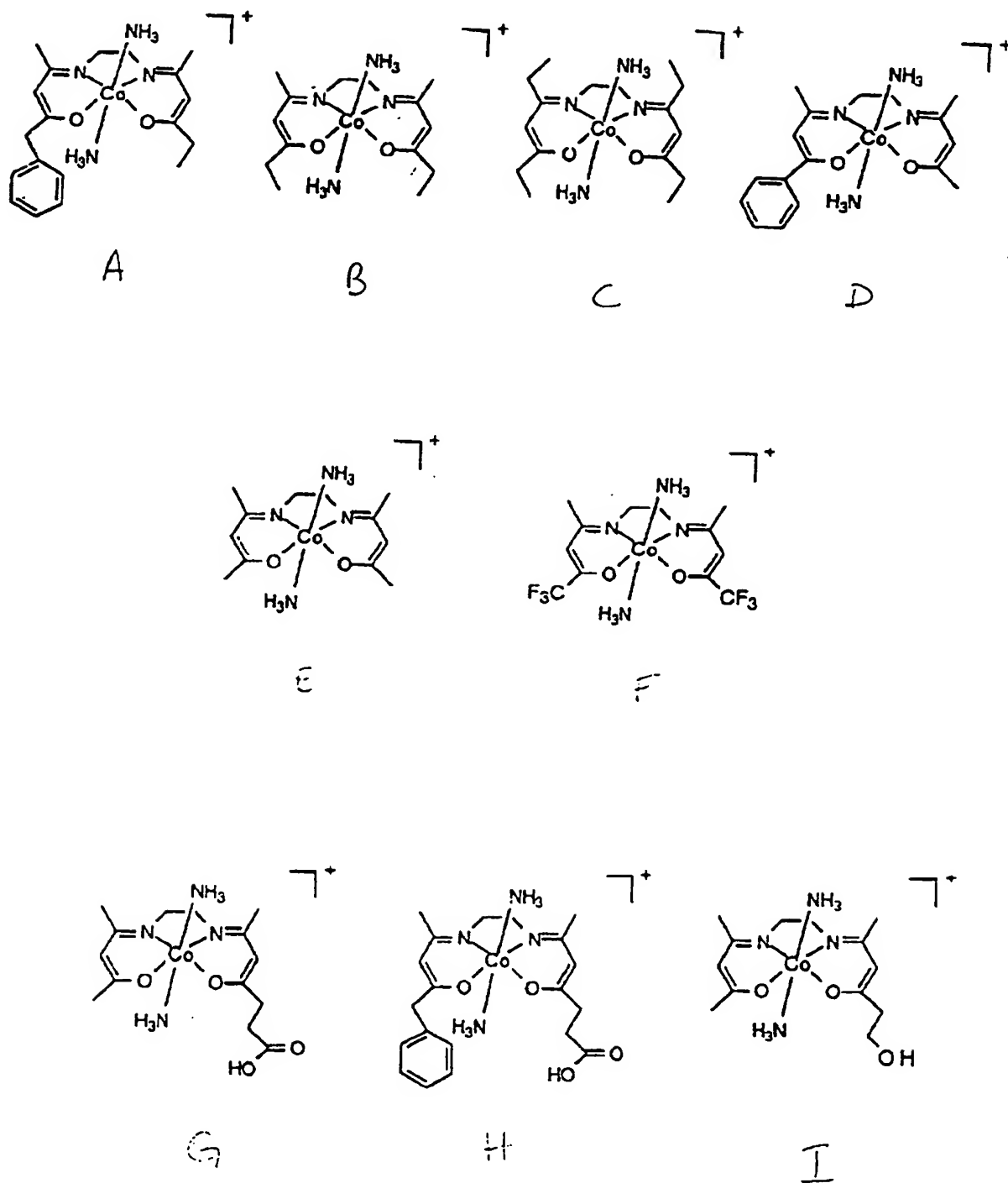


Figure 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/19900

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.65, 1.69, 1.73, 646, dig.6; 435/214; 514/6, 23, 44, 129, 501; 530/345, 400, 409; 536/23.1, 121; 556/13, 32, 138, 146

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, DIALOG

search terms: schiff, cobalt, chelate, tetradentate, conjugate, target, zinc finger, enzyme, thrombin, structure of claim 1

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,514,522 A (SIEVERS ET AL.) 30 April 1985 (30.04.85), column 2, lines 12-44.	1-15
X	US 4,948,506 A (LONSDALE ET AL.) 14 August 1990 (14.08.90), column 16, lines 32-39, column 17, lines 37-50.	1, 2, 6, 14
A	US 5,142,076 A (DORI ET AL.) 25 August 1992 (25.08.92), column 2, lines 36-66.	1-15
X,P	WO 96/18402 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 20 June 1996 (20.06.96), see entire document.	1-15

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be of particular relevance	*X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means	
*P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

25 FEBRUARY 1997

Date of mailing of the international search report

26 MAR 1997

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19900

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	BHATTACHARYA et al. Ambient Oxygen Activating Water Soluble Cobalt-Salen Complex for DNA Cleavage. Journal of the Chemical Society, Chemical Communications. 21 December 1995, Number 24, pages 2489-2490.	1-15
A	Chem. abstr., Volume 83, Number 2, 14 July 1975 (Columbus, OH, USA), page 761 column 2, the abstract No. 21107u, FUJII, Y. 'Preparation and properties of cobalt(III)-Schiff base complexes containing optically active ligands at the axial sites.' J. Sci. Hiroshima Univ. Ser. A: Phys. Chem. 1974, 38(2-3), 313-326 (Eng.).	1-15
A	Chem. abstr., Volume 101, No. 26, 24 December 1984 (Columbus, OH, USA), page 7, column 1, the abstract No. 101:231134s, SPIRATOS et al. 'Coordination polymers. VI. Preparation and characterization of some azomethine chelate polymers.' Rev. Roum. Chim. 1984, 29(5), 457-460 (Eng).	1-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/19900

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 31/045, 31/13, 31/19, 31/295, 31/70, 38/02; C07F 15/06; C07H 21/00, 23/00; C07K 2/00; C12N 9/74

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/646; 435/214; 514/6, 23, 44, 129, 501; 530/345, 400, 409; 536/23.1, 121; 556/13, 32, 138, 146